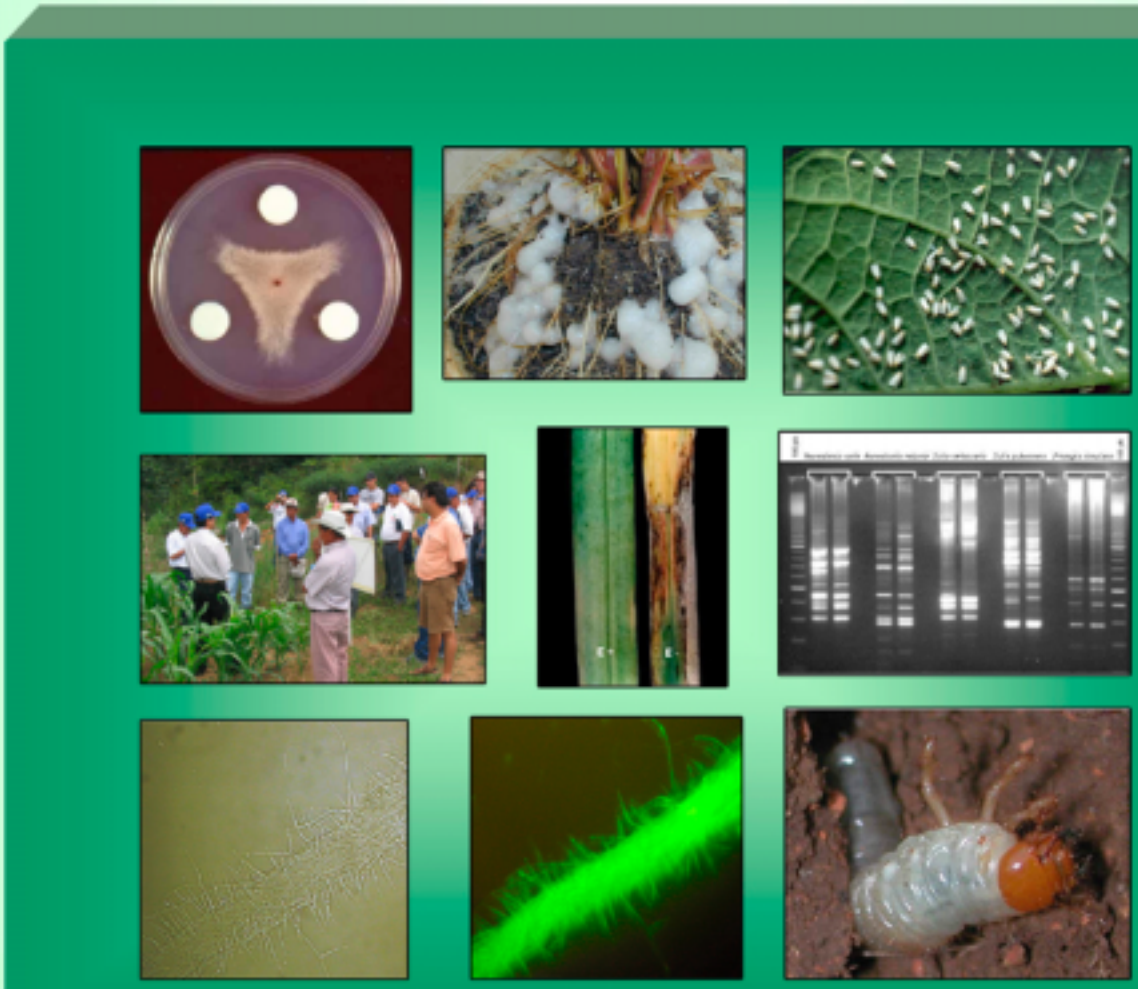
Crop and Agroecosystem Health Management

Project PE-I

Annual Report 2005

CIAT
Centro Internacional de Agricultura Tropical
International Center for Tropical Agriculture



Crop and Agroecosystem Health Management

Project PE-1

Annual Report 2005

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Crop and Agroecosystem Health Management (Project PE-1)

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Cover photos: From left top row: 1) Fungal growth inhibition by antifungal protein from *Clitoria* seeds, 2) Spittlebugs in *Brachiaria* , 3) High population of whiteflies *(Bemisia tabaci)* on bean plant; middle row: 1) Field day with farmers in Pescador, Colombia discussing the economic threshold of white grubs on maize, 2) Disease resistance conferred by the presence of endophytic fungus in *Brachiaria,* 3) Molecular detection and differentiation of spittlebug species; bottom row: 1) Mycelium of the endophytic fungus *Aremonium implicatum,* 2) Mycelium of the endophytic fungus *Aremonium implicatum* expressing the green fluorescent protein gene, 3) Larvae of *Phyllophaga mentriesi*.

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Project overview: PE-1: Crop and agroecosystem health management 2005-2008

Project Manager: Segenet Kelemu

Project Description:

Goal: Enhance crop yields and quality of products, reduce pesticide use and residue, and improve agro-ecosystem health through enhancement of integrated management of major pests and diseases in the tropics and soil health.

Objective: Develop and transfer pest-and-disease knowledge and management systems for sustainable productivity and healthier agro-ecosystems in the tropics.

Important Assumptions: 1) Donor support to projects; 2) Active collaboration from other IARCs and other research organizations; 3) Active collaboration from CIAT's projects (e.g. TSBF); 4) Active participation from NARS.

Target Ecoregion: Humid and sub-humid tropics in eastern and southern Africa, Central America and Andes.

Beneficiaries and End Users: Information on biodiversity in tropical agroecosystems, improved IPM components and technologies and knowledge systems will benefit NARS scientists, extension workers, farmers and consumers, by increasing crop yields, crop quality, agro-ecosystem health and stabilizing production systems.

Collaborators: International Agricultural Centers through the System wide program on Integrated Disease and Pest Management, NARS Latin America (eg. CORPOICA, Colombia; EMBRAPA, Brazil; INIFAP, Mexico; DICTA, Honduras) and Africa (e.g.NARO, Uganda; EARO, Ethiopia; ISAR, Rwanda), universities (eg. Cornell, University of Kentucky, Kansas State University, University of Florida, Universidad Nacional, Universidad Valle, Alemaya, Makerere and Nairobi Universities, U. Nacional de Costa Rica, etc.), private sector (eg. BioTropico, ASCOFLORES) NGOs (eg. Manrecur)

Project changes: The Integrated Pest and Disease Management project has made the following changes in 2005:

Previous Project Name: Integrated Disease and Pest Management

Comment: The various activities and outcomes of the project focuses not only on crop health in general through host resistance, conservation and utilization of natural resources (such as natural enemies and other biocontrol agents, plant and microbial derived biopesticides), judicious use of pesticides, and other novel strategies of disease and pest management, but also on general soil health. These measures in turn contribute to agroecosystem health (human, wildlife, soil, water, beneficial organisms, etc.) due to reduction in indiscriminate use of pesticides. Not only increased crop yields are achieved, but also enhanced quality of products (eg. products with low or no pesticide residues) that benefit producers and consumers; and healthier environment can result from development and implementation of environmentally-friendly disease and pest management strategies.

New Project Name: Crop and Agroecosystem Health Management

Previous Goal: To increase crop yields and reduce environmental contamination through the effective management of major pests and diseases.

Comment: The project will focus on strategies to enhance soil health (developing strong ties with TSBF-CIAT), host resistance, biopesticides and other novel methods of disease and pest management strategies in order to enhance crop yields and quality of products, as well as improve agro ecosystem health in general. We will seek to apply environmentally-friendly disease and pest management strategies to non-CIAT commodities in the tropics, particularly to African crops. Because we have over the years developed many tools and methods for disease and pest diagnosis, detection, control strategies mainly on CIAT commodities, great efforts would be made to apply these technologies to crops such as fruits, vegetables and other high value crops. We plan to explore ways of enhancing incomes of small producers through products with little or no pesticide residues (eg. organic farming).

New Goal: To enhance crop yields and quality of products, reduce pesticide use and residue, and improve agro-ecosystem health through enhancement of soil health and integrated management of major pests and diseases in the tropics.

Previous Output 3: NARS' capacity to design and execute IPM research and implementation strengthened.

Comment: Many of the project scientists and their support staff are well-trained molecular biologists who develop and apply various molecular tools for the detection, characterization and diagnosis of pests and diseases; clone genes from various organisms, sequence genomes of organisms, apply recombinant DNA and transgenic technologies for disease and pest management, as well as train various NARS scientists and students in molecular tools and procedures. Therefore, capacity building of NARS in these important areas of research (which are in demand particularly in Africa) is added to this output.

New Output 3: NARS' capacity to design and execute IPM research and implementation, and applications of molecular tools for pathogen and pest detection, diagnosis, diversity studies as well as novel disease and pest management strategies strengthened.

CIAT: PE-1 Project Log Frame (2005-2007)

Project: Crop and agroecosystem health management
Project Manager: Segenet Kelemu

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
Goal To enhance crop yields and quality of products, reduce pesticide use and residue, and improve agro-ecosystem health through enhancement of soil health and integrated management of major pests and diseases in the tropics.	∉ % increased in crop yields. ∉ % reductions in pesticide use and prevention of environmental degradation through adoption of improved technology. ∉ % reduction of losses to several major diseases and pests.	∉ Production statistics. ∉ Adoption and impact studies. ∉ Project reports.	∉ National policies favorable to adoption of IPM strategies (i.e., increased support to extension, reduction of subsidies for pesticides). ∉ National programs are active and strong in key countries.
Purpose To develop and transfer pest-and-disease knowledge and management systems for sustainable productivity and healthier agro-ecosystems.	∉ Number of new cultivars with resistance to pests and pathogens released and used by farmers. ∉ Number of released and established bio-control agents. ∉ Number of environmentally friendly control strategies adopted by farmers.	∉ Adoption and impact studies. ∉ Performance of new cultivars ∉ End-of-project reports. ∉ Refereed publications, book chapters.	∉ Donor support to projects. ∉ Active collaboration from other IARCs and other research organizations. ∉ Active collaboration from CIAT's projects (e.g. TSBF).
Output 1 Pest and disease complexes described and analyzed.	2005 ∉ Reduction in cassava whitefly damage. Colonies of homopteran (1 or 2) species established. ∉ Biology determined (1species). ∉ Transmission studies carried out. ∉ Taxonomic identification of white grub and burrower bug species. ∉ Detection of endophytic fungi in *Brachiaria* and distribution determined. ∉ A set of microsatellite markers associated with blast resistance genes identified. ∉ A set of 20 rice lines with tolerance to sheath blight identified.	∉ All areas: project reports, refereed publications, book chapters. ∉ Reports with maps, economic damage, biological information. ∉ Analysis of experiments. ∉ Transfer of tools to seed health facilities. ∉ Molecular markers for pest and diseases available. ∉ Candidate genes for resistance identified.	∉ NARS have the needed resources. ∉ Adequate interaction with other disciplinary scientists. ∉ Successful experiments. ∉ Continued development of new varieties that are commercially acceptable. ∉ Farmers have adequate access to extension agents, credit lines, and other factors that influence adoption. ∉ Collaboration with NARS possible. ∉ Evaluation, screening, and exploration sites accessible.

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
Output 2 Pest-and-disease management components and IPM strategies developed.	2005 ∉ Taxonomic identification entomopathogenic fungi, bacteria or nematodes; data from laboratory experiments available. Publications in journals. ∉ Analysis of field and greenhouse data. ∉ Levels of resistance confirmed in bean progenies. ∉ Five to ten tolerant bean varieties selected in farmers' fields and greenhouse evaluation. Experimental data available; resistant bean lines identified. ∉ Set of rice blast isolates with a virulence genes for corresponding resistance genes. ∉ Extension service providers (50), and farmers (300) trained in Bean IPDM in Uganda and Rwanda. ∉ A list of sources of CFSD resistance will be available. The work published in the annual report and a journal paper. 2006 ∉ Agreement with commercial biopesticide industry established for product development. ∉ Two commercial *Brachiaria* cultivars with spittlebug resistance available to farmers. ∉ Levels of disease and insect resistance confirmed in bean genotypes ∉ 500 common bean farmers in Malawi, 1500 in Kenya and 8000 in Tanzania evaluated botanical biopesticides and other pest management options. ∉ Distribution of rice nurseries with 50 potential donors of blast and sheath blight resistance to Latin American countries. ∉ Studies on enhancement of general soil health initiated. 2007 ∉ Three to four biological pesticides commercially available for farmers. ∉ Foliar blight resistant *Brachiaria* hybrids available.	∉ Analysis of experiments. ∉ Guidelines for IPM. ∉ Reports on field effectiveness and probability of adoption of components. ∉ Field-oriented brochures. ∉ Farmer participatory research implemented. ∉ Reports available.	Funding for research and technology (IPM) practices available. Stakeholders are willing to participate.

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
	∉ Multiplication and distribution of Latin American rice cultivars with complementary blast resistance genes incorporated. ∉ Implementation of marker assisted selection breeding program for sheath blight resistance in rice. ∉ Practices to control Moko of banana validated by selected banana farmers in Colombia. ∉ Research partners in Kenya and Rwanda trained and collaborate in *Pythium* root rot assays on beans. ∉ Angular leaf spot and *Pythium* resistant bean varieties available to Bean Farmers. ∉ The populations for genetic studies developed and F2 population analyzed for disease susceptibility and molecular markers. ∉ Results of study using several CTV mild strains to determine if they provide adequate protection.		
Output 3 NARS' capacity to design and execute IPM research and implementation, and applications of molecular tools for pathogen and pest detection, diagnosis, diversity studies as well as novel disease and pest management strategies strengthened.	2005 ∉ Five hundred farmers in Malawi, 1500 in Kenya, 8000 in Tanzania and 1000 in Uganda evaluated biopesticide and other pest management practices on common bean crop. 2006 ∉ Cassava, maize and onion farmers trained in management of soil-borne pests (white grubs and burrowers bugs). ∉ Molecular markers associated with resistance identified in rice. ∉ Molecular tools for detection, diagnosis and diversity studies of a number of pathogens and pests made available.	∉ Reports on training courses. ∉ Concept notes and projects prepared with partners. ∉ IPM projects implemented	Trainees are keen to become trainers of farmer communities.

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
	∉ Isolates of *Fusarium* and *Pythium* pathogens in beans characterized and identified to species. ∉ Work on anthracnose diseases of tropical fruits initiated. 2006 ∉ AFLP fingerprints for *C. lindemuthiuanum* generated. ∉ Virulence level among isolates of *R. solani* infecting *Brachiaria* described. ∉ Practices to control *Phytophthora* root rot (PRR) validated by selected Colombian cassava farmers. ∉ Isolates of *Pythium* pathogens on beans characterized and identified. Data available for publication. ∉ Biocidal proteins from tropical forages isolated and characterized. ∉ Better diagnostic method and more information on the virus published in the annual report and journal paper. ∉ Diagnostic tools for citrus psorosis virus and citrus leprosis virus developed and available for certification programs. ∉ The anthracnose pathogen population of tropical fruits characterized in regions of Colombia. 2007 ∉ RAMS and AFLP data for *C. lindemuthiauanum* available. ∉ Antifungal protein gene identified and available in tropical forages. ∉ DNA sequences in gene bank for resistance to cassava frog skin disease reported and published.		

Narrative Summary	Measurable Indicators	Means of Verification	Important Assumptions
	2007 ≤ Combination of whitefly resistant cassava varieties and biological control agents available to farmers and farmers trained. ≤ Application of biopesticides and cultural practices by farmers. ≤ Hundred or more bean farmers and technicians trained in whitefly management. ≤ Combination of pest resistant bean varieties and biological control agents available to farmers and farmers trained. ≤ Awareness of IPM in beans created among policy makers and other stake holders (NGO's, private sector, schools, etc.). Farmers meetings, field days, exchange visits, publication of promotional material.		
Output 4 Global IPM networks (Integrated Whitefly Management Technology) and knowledge systems developed.	≤ Network of researchers established. ≤ Preparation of Web pages and databases with relevant IPM information. ≤ Databases of microbial and arthropod collection established.	≤ Electronically published Web pages and databases. ≤ Progress reports.	

MTP 2006-2008
Project Logframe
PE-1

	Outputs	Intended User	Outcome	Impact
Output 1	Pest and pathogen complexes described and analyzed.	NARS and university researchers, extension workers, students, CIAT scientists	Molecular and conventional tools for disease and pest diagnosis, detection and characterization developed, and applied to better understand insect pest and pathogen complexes	Improved stable crop productivity from better disease and insect pest management strategies.
Output 1 Targets 2006	∉ Taxonomic identification of invasive pest species and white grub and burrower bug species on CIAT commodities and related agroecosystems. ∉ Taxonomic identification entomopathogenic fungi, bacteria or nematodes; and cassava whitefly natural enemies in key regions determined. ∉ *Fusarium* and *Pythium* pathogens of beans and five natural enemies of soil pests identified and characterized	NARS researchers in LAC, Asia and Africa, CIAT scientists	Microbial and insect pest characterization tools developed.	Improved disease and insect pest management strategies.
Output 1 Targets 2007	∉ Anthracnose pathogens of beans and tropical fruits from Colombia characterized and the virulence level of *R. solani* isolates from *Brachiaria* and rice determined. ∉ Diagnostic tools for two citrus viruses (psorosis and citrus leprosies) and cassava frogskin disease developed and made available. ∉ Molecular tools for detection, diagnosis and diversity studies of a number of pathogens and pests made available.	Researchers in LAC, Asia and Africa, CIAT scientists	Adoption of disease and pest characterization tools.	More efficient and accurate tools for disease and pest diagnosis; improved understanding of disease and pest complexes
Output 1 Targets 2008	∉ Two plant growth promoting bacteria and one biological control agent characterized.	Researchers in LAC, Asia and Africa	New options for disease and pest management and plant health enhancement; tools for disease and pest characterization.	Diversity of options for enhancing plant health.

PE-1 Project logframe MTP 2006-2006 (cont'd)

	Outputs	Intended User	Outcome	Impact
Output 2	Pest-and-disease management components and strategies developed.	Researchers in LAC, Asia and Africa	Disease and pest resistant lines; disease and pest management strategies	Increased crop yields and enhanced quality of products; increased and stable income.
Output 2 Targets 2006	∉ A set of microsatellite markers associated with rice blast resistance genes identified. ∉ Bean, cassava, rice and tropical forage lines resistant to major diseases and pests identified. ∉ Biocidal proteins from tropical forages isolated and characterized. ∉ Cultural practices that enhance soil health and control soil pests validated by selected farmers in Colombia. ∉ Biopesticide for cassava whiteflies evaluated.	Researchers in LAC, Asia and Africa; CIAT scientists	Disease and insect pest resistant bean, cassava, rice and tropical forage lines.	Increased and stable yields.
Output 2 Targets 2007	∉ Two commercial *Brachiaria* cultivars with spittlebug resistance available to farmers; Whitefly resistant cassava variety available to farmers; 50 blast and sheath blight resistant rice lines distributed to Latin American countries and marker assisted selection implemented for 3 diseases. ∉ Efficacy of cassava whitefly parasitoids determined.	Researchers in LAC, Asia and Africa; CIAT scientists; farmers	Disease and pest resistant genotypes made available	Enhanced and stable productivity
Output 2 Targets 2008	∉ Three biological pesticides commercially available; an antifungal protein gene from tropical forages available and used in other crops.	Researchers in LAC, Asia and Africa; farmers	Disease /pest resistant crops made available; biopesticides made available	Enhanced and stable productivity, healthier environment.
	∉ Foliar blight resistant *Brachiaria* hybrids available.			
	∉ Multiplication and distribution of Latin American rice cultivars with complementary blast resistance genes			

PE-1 Project logframe MTP 2006-2006 (cont'd)

	Outputs	Intended User	Outcome	Impact
	∉ Cultural practices to control Moko of banana validated by selected banana farmers in Colombia			
	∉ Angular leaf spot and *Pythium* resistant bean varieties available to bean farmers.			
Output 3	NARS' capacity to design and execute IPM research and implementation, and applications of molecular tools for pathogen and pest detection, diagnosis, diversity studies as well as novel disease and pest management strategies strengthened.	NARS in LAC, Asia and Africa; farmers	Improved capacity for disease and pest management strategies and knowledge on new molecular tools.	Efficient tools for disease and pest diagnosis; environmentally-friendly disease and pest management strategies.
Output 3 Targets 2006	∉ Facilitate partners with a goal to train five hundred farmers in Malawi, 1500 in Kenya, 8000 in Tanzania and 1000 in Uganda, for evaluation of biopesticide and other pest management practices on common bean crop. ∉ Extension service providers (50), and farmers (300) trained in Bean integrated pest and disease management in Uganda and Rwanda. ∉ Research partners in Kenya and Rwanda trained and collaborate in *Pythium* root rot assays on beans. ∉ Whitefly IPM components validated with cassava producers.	NARS and farmers in Africa	Options for disease and pest management strategies	
Output 3 Targets 2007	∉ Cassava, maize and onion farmers trained in management of soil-borne pests (white grubs and burrowers bugs). ∉ Cassava farmers trained in whitefly IPM tactics.	NARS and farmers in LAC, Africa		
Output 3 Targets 2008	∉ Combination of whitefly resistant cassava varieties and biological control agents available to farmers and farmers trained. ∉ Hundred or more bean farmers and technicians trained in whitefly management.	NARS, NGOs and farmers	Improved disease and pest management practices.	Stable and increased yield and quality.

PE-1 Project logframe MTP 2006-2006 (cont'd)

	Outputs	Intended User	Outcome	Impact
	∉ Cassava whitefly IPM introduced to countries in LA.			
Output 4	Global IPM networks and knowledge systems developed.	NARS, NGOs, universities, and farmers	Improved communications and exchange of information and materials	Improved access to information; sharing of natural resources such as beneficial organisms
Output 4 Targets 2006	∉ Network of researchers established. ∉ Databases of microbial and arthropod collection established.			
Output 4 Targets 2007	∉ Preparation of Web pages and databases with relevant IPM information.			
Output 4 Targets 2008	∉ Preparation of laboratory manuals			

Output 1: Pest and pathogen complexes in key crops described and analyzed.

Activity 1.1. Rapid identification of *Colletotrichum lindemuthianum*-specific microsatellite markers using 5'anchored PCR.

Contributors: G. Mahuku; M.A. Henriquez and M. Navia

Highlight:

- We identified and developed primers specific to 16 microsatellite loci in the bean anthracnose pathogen, *Colletotrichum lindemuthianum*, and showed the potential of these primers to distinguish between Andean and Mesoamerican groups and to provide information on the genetic structure of the pathogen.

Rationale

Knowledge of the genetic structure of plant pathogen populations is important because the amount of genetic variation that is maintained in a population reflects the capacity of a pathogen to evolve, information which is important in choosing the most effective control strategy (Burdon, 1993, Genetic Variation in pathogens populations and its implications for adaptation to host resistance, In: Durability of Disease resistance (Jacobs, T., Parleviet [eds]), Kluwer, Dordrecht, pp 41-56; McDonald & McDermott, 1993, Bioscience 43: 311-319). The distribution of genetic variation can be used to select the most effective resistance gene(s) that are better suited to manage pathogen populations in a given locality. Several tools have been used to characterize *C. lindemuthianum*, but the lack of a standardized molecular system makes it impossible to compare data coming from different laboratories and to have a concerted (unified) effort (strategy) for developing anthracnose management strategies. A standard set of differential cultivars is used. Although this has greatly facilitated comparison of data, the process is long and tedious, and subject to environmental conditions. Where conditions for inoculum preparation, inoculation and humidity are not controlled, the procedure is not precise. Several types of molecular markers have been used, but there are disadvantages with many of these. For example, although RAPD-PCR has high resolution, and is simple to carry out, it requires exquisite control to achieve robustness. AFLPs are also very informative but require careful optimization of conditions for restriction enzyme activity, ligation of adapters and PCR. Microsatellites are tandemly repeated copies of short nucleotide sequences that are useful for PCR-based DNA typing of fungi. Once developed, microsatellites behave like RAPD markers with the added advantage of robustness. We used the 5' anchored PCR method (Fisher *et al*., 1996, Nucleic Acid Research 24: 4369-4371), to identify microsatellite loci without the expenses of library screening. We report the isolation and characterization of microsatellite loci in the bean anthracnose pathogen *C. lindemuthianum*.

Materials and Methods

Fungal isolates and DNA extraction: Four *C. lindemuthianum* isolates previously characterized on a set of 12 anthracnose differential varieties and classified as Andean (two) and Mesoamerican (two), were used in this study. Isolates were recuperated from lyophilizaed samples. Mycelia production and DNA extraction was according to the method described by Mahuku *et al.* (2004, Plant Molecular Biology Reporter 22: 71-81). The DNA quality was assessed by electrophoresis in a 0.7% agarose gel and the quantity measured using the fluorometer (Hoefer® DyNA Quant 2000, Pharmacia Biotech, USA) and adjusted to 10 ng/µl in 0.1X TE buffer. A further 35 isolates

representative collected from Andean and Mesoamerican regions were used to standardize cycling protocol and test the suitability of developed microsatellites.

RAMs PCR, sequencing and microsatellite identification: A series of degenerate di – and tri - nucleotides microsatellite primers anchored at the 5' end (Table 1.1.1), were used to amplify genomic DNA from two Andean and two Misoamerican isolates. The 3'end of each primer was complemenatry to a common fungal repetitive sequence whilst the 5'end provided a degenarate anchor sequence to avoid slippage. The rationale behind this design was that if the primer annealed to two close and inverted simple sequences repeats, then the region between them, which may also contain repetitive sequences would be amplified. The conditions of the PCR amplification were according to Ganley and Bradshaw (Ganley and Bradshaw, 2001, Mycological Research 105: 1075-1078), in an MJ100 PTC model thermal cycler. PCR products were separatyed on 1.5% agarose gels. For each degenerate primer, descrite bands in the 600-1200 bp region were excised from the gels, gel purified with a QiaEX II gel Extraction Kit (QIAGEN) and cloned with a pGEM easy vector kit (INVITROGEN). Plasmid DNA was obtained and sequnced using the ABI PRISM™ 377 DNA automated sequencer (Perkin Elmer). DNA sequences were analysed using Seqman within DNAStar (DNAStar, Madison, WIS. USA) and PCR products containing repeated sequences were selected. Specific primers flanking the region of microsatellites were designed using Primer3 software (Center for Genome Research, Whitehead Institute, MA, USA - http://www-genome.wi.mit.edu/cgi-bin/primer/primer3), and commercially synthesized (Integrated DNA Technologies, Inc., Coralville, IA, USA). These primers were tested first on four isolates and on an additional set of 35 isolates representative of Andean and Mesoamerican groups.

Table 1.1.1. 5'anchored primer sequences (5'to 3').

ID	Sequence
PTC1	KKYHYHY$(GA)_6$
PTC3	KKYNSSH$(AAG)_5$
PTC4	KKVRVRV$(CT)_6$
PTC5	KKVRVRV$(TG)_6$
PTC6	KKBNVSS$(GATA)_4$

Results and Discussion

Amplification of four *C. lindemuthianum* isolates using the anchored primers gave distinct 'RAPD-type' DNA profiles; and example with the anchored PTC1, PTC4 and PTC5 primers are shown in Figure 1.1.1. No products were observed for PTC3 and PTC6 primers (Figure 1.1.1). Reproducible profiles were seen in replicate tests for all primers. Potentially informative microsatellite loci were identified by selecting anchored-PCR products from gels on the basis of their consistent amplification or their apparent uniqueness to an individual isolate. Thirty such PCR products were purified, cloned and sequenced.

Sixteen of the cloned microsatellite loci were chosen for further assessment on the basis of the size of the microsatellite repeat or the number of repetitive sequences present. For each of these a locus-specific primer was designed so that a PCR product of approximately 100-500 bp in length would be obtained when used in conjunction with the anchored primer or in pairs. Seventeen

primer pair combinations were obtained; eight of which gave single PCR products, one gave two, one gave four, and six consistently gave multiple PCR products (Figure and Table 1.1.2).

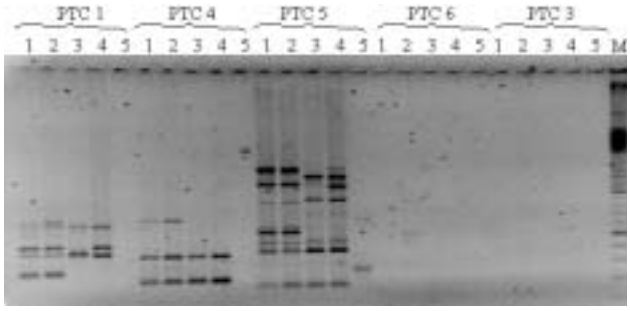


Figure 1.1.1. PCR amplification products from *Colletotrichum lindemuthianum* isolates obtained using the anchored PTC1, PTC3, PTC4, PTC5, and PTC6 primers. Lane 1 and 2 are *C. lindemuthianum* Andean isolates while lanes 3 and 4 a Mesoamerican isolates. Lane 5 is negative control (no fungal DNA was added) and lane M is the 100 bp molecular size marker. NO products were obtained with PTC3 and PTC6.

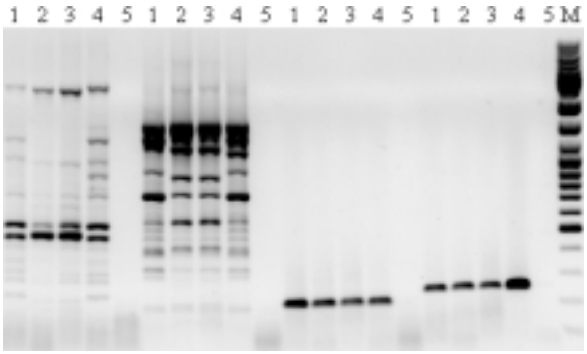


Figure 1.1.2. Microsatellite length variation between Andean and Mesoamerican isolates of *Colletotrichum lindemuthianum* isolates. Lanes 1 and 2 are Andean isolates; lanes 3 and 4 are Mesoamerican isolates, lane 5 is negative control (no DNA added), lane M is 100 bp molecular size marker.

We are in the process of optimizing the annealing temperature so that single fragments are consistently amplified, after which we will test promising microsatellite primers on 35 Andean and Mesoamerican isolates from different regions where anthracnose of common bean is a serious problem. Preliminary evaluation of nine *C. lindemuthianum* isolates collected from Andean and Mesoamerican beans and regions using primers specific to the Anth.CL_006 loci revealed four alleles (Figure 1.1.3), while amplification of 35 isolates from a world-wide collection using Anth.CL001 primer pair revealed two alleles, one that was consistently associated with isolates belonging to the Andean group, and another of isolates belonging to the Mesoamerican group (Figure 1.1.4). These preliminary results show the potential of these markers to distinguish between Andean and Mesoamerican groups and for population genetic studies.

Table 1.1.2 Polymorphic microsatellite loci for the bean anthracnose pathogen, *Colletotrichum lindemuthianum*.

Locus	Primer sequence 5' to 3' Forward	Reverse	# of alleles	Repeat type	size range
Anth.CL001	TGTCACAGGGGGGGG	GGCAGAACCAGGATG	multiple	PTC1	271
Anth.CL002	TGGGCTCTCTCTCTCTTCC	GGTGGGAGAA	2	PTC4	4
Anth.CL003	GGCTCTCTCTCTCT	CAGGAACAAACAGGG	multiple	PTC4	234
Anth.CL004	TCATGCTACTCTGACTCCTGG	GAGCGCTCTGC	multiple	PTC4	400
Anth.CL005	CCCTCAACCTCTTTGTGG G	GAGCGCTCTGC	PTC4	234	
Anth.CL006	TGGGATCCTGATGATG	GAGTGGTATAATCTG	2	PTC 5	270
Anth.CL007	GGTGTCTCGTACCTA	ACAAATGGAAGCG	2	PTC5	239
Anth.CL008	TGTGTATCAACGCCT ACCCCGTATCATACG		4	PTC5	234, 1200
Anth.CL009	TCTCTAACAGGG GAAG	GGGGGGGGGG	multiple	PTC5	274
Anth.CL010	TAGGACAGCGC GCATCGTCTGCACAT		2	PTC5	289
Anth.CL011	GTGGGGGGTG ATGTTCGTTGGTCTC		multiple	PTC5	641
Anth.CL012	TACCTATCTGCGAG GGGGGTGGAG		2	PTC5	335
Anth.CL013	GGTGGGGGG GAGTCCGTCACATCAA		4	PTC5	500
Anth.CL014	TCAGGGGG TGGG GCCATC TTTGATGCTG		multiple	PTC5	184
Anth.CL015	GATACAAAGTG CATG ACCCCTAGCGTGT		2	PTC5	340
Anth.CL016	GATAAAGCCTG CAAA	GCAGCACAGCACTTCTCG	2	PTC5	248

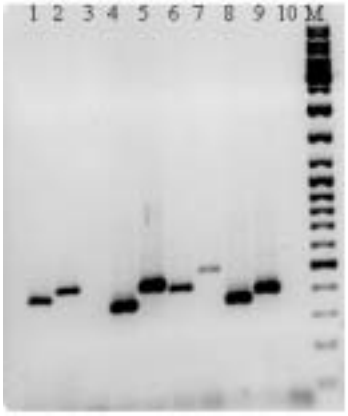


Figure 1.1.3. PCR amplification of nine *Colletotrichum lindemuthianum* isolates of a diverse geographical origin using primers specific to the Anth.CL006 primers. Lanes 1, 3, 4, 7, and 8 are *C. lindemuthianum* isolates collected from Mesoamerican genotypes while lanes 2, 5, 6, and 9 represent isolates collected from Andean genotypes. Lane 10 is negative control while lane M is the 100 bp molecular size marker.

Figure 1.1.4. Amplification of the SSR markers corresponding to specific Anth.CL001 locus from genomic DNA of 35 Colleto*trichum lindemuthianum* isolates collected from Andean and Mesoamerican bean genotypes and regions shown on a silver-stained polyacrylamide gel.

Conclusions: Primers specific to 16 microsatellite loci in *C. lindemuthianum* were developed. Six of these primers consistently gave multiple fragments. Preliminary evaluation of nine and 35 isolates using primers specific to two microsatellite loci revealed the potential of these SSR markers to distinguish between different *C. lindemuthianum* isolates and to generate information that can be used to infer the genetic structure of this fungus. We are currently optimizing the cycling conditions for these primers, and using them to characterize a set of 120 *C. lindemuthianum* isolates that have been collected from different regions of Colombia.

Activity 1.2. Develop multiplex PCR assay for simultaneous detection of 6 *Pythium* species in common bean soils.

Contributors: G. Mahuku and R. Buruchara

Highlight:

- Developed a multiplex PCR assay for simultaneous detection of six pathogenic *Pythium* species and a potential biocontrol agent.

Rationale

Root rots are an increase problem to bean production in East Africa that has resulted in 100 % yield losses, and in some cases, (e.g. western Kenya) farmers were forced out of bean production. Previous characterization work identified *Pythium* as the most important causative agent and six species (*P. ultimum,P. spinosum,P. deliense,P. salpingophorum,P. torulosum* and *P. nodosum*) were shown to be important in inciting root rots of bean. Of these species, *P. ultimum* var *ultimum* is the most prevalent and devastating. However, identifying and distinguishing between these species, using morphological or pathogenic characteristics is difficult and slow. In the soil, these species may be mixed with a range of other host pathogens and saprophytes (e.g. Mortilela spp. that is morphologically indistinguishable from Pythium). Detection and identification of the causative species is crucial for identifying resistance or introducing and targeting other disease management practices. This study was initiated to develop a multiplex PCR assay for the simultaneous detection and identification of the major *Pythium* species associated with common bean root rots. Multiplex PCR is a method for simultaneous amplification of several fragments in a single PCR and is one of the best molecular tools for species identification, as it enables the identification of several species by a single PCR followed by a single electrophoresis.

Materials and Methods

Primer design: The internal transcribed spacer region 1 (ITS 1) of the ribosomal DNA was targeted for the development of *Pythium* species-specific primers. DNA sequences from our own work (CIAT 2003, Bean Project Annual Report), and from the data bank of Dr Andre Levesque (AAFC Canada) were compared for the 6 target *Pythium* species, and based on sequence differences in the ITS 1 region, species-specific primers were designed. We also took note of the primers that had been developed and reported by other groups (Table 1.2.1), and where these matched what we had designed, the same primer sequences were synthesized. Where there were differences from our optimized sequences, the two primers, our own and the one reported were synthesized and tested for specificity by amplifying test strains.

Primer optimization : The specificity of the primers to the target strain was tested by amplifying DNA of several *Pythium* species that we have in our collection. Once found to be specific, the primers were optimized, to select suitable primer combination that avoid formation of heterodimers, before optimizing for magnesium concentrations, DNA concentrations, enzyme concentrations, annealing temperature and cycling and electrophoresis conditions so as to enable simultaneous detection of the target species.

Suitability for detecting different Pythium species: Two approaches were followed to test the suitability of the multiplex PCR assay for simultaneous detection of different *Pythium* spp: (1) primers were mixed, added one at a time and used to amplify DNA from individual species, until all primers were part of the mix; (2) DNA from different species were mixed and amplified, first with individual primers, then with different primer combinations.

Results and Discussion

Multiplex PCR assay for Pythium species: PCR primers specific to six *Pythium* species, including *P. oligandrum*, a known biocontrol agent designed based on sequences differences in the internal transcribed spacer region 1 of the ribosomal DNA were used in conjunction with an oomycete specific primer that was previously developed by Dr. Andre Levesque, AAFC, Canada. The Oomycete specific primer is based on conserved sequences in the 5.8S ribosomal genes. The designed primers, their sequences and the target fragment size are shown in Table 1.2.1.

Suitability for multiplexing: When DNA from different *Pythium* species was mixed at a concentration of 10 ng each, and used as template in PCR reactions with specific primers, only the target species was amplified in all cases, (Figure 1.2.1). Mixing primers of all species to a final concentration of 0.4 µM, and adding DNA from individual *Pythium* species resulted in the amplification of the desired fragment (Figure 1.2.2). Two fragments the desired 150 bp and a ~400 bp fragments were amplified *P. oligandrum* (Figure 1.2.2). Increasing the PCR buffer concentration to 1.5 X and annealing temperature to 65°C resulted in the disappearance of the larger fragment. The annealing temperatures for the different primers ranged from 56 – 70, and as such, the multiplexing should take this into consideration.

Table 1.2.1. Amplification results from the designed primers using genomic DNA from different *Pythium* species.

Species	Primer ID	Size (bp)	Sequence 5' to 3'	Source
P. spinosum	Pspi (old)	200	TGT GTG TTG TGA TCG TGC CT	Wang *et al*., 2003, Lett. Appl. Microb.37: 127-132
P. spinosum	Psp1 (new)	325	TGT TGT GTG TCT GCG CCG TTG TTG G	This project
P. ultimum	PuK1	192	ACG AAG GTT GGT CTG TTG	Kageyama *et al*., 1997, Plant Dis. 81:1155-1160
P. deliense	Pdel1	182	GCT GAA CGA AGG TGG GCT GCT	This project
P. salpingophorum	Psal1	217	TTA TGT TCT GTG CCT TCT CTC G	This project
P. oligandrum	Po1	150	TGC GTC TAT TTT GGA TGC GG	Wang et al., 2003
P. nodosum	Pnod1	232	ATC TGC TCT CTG TGC CTT TCG	This project
P. torulosum	Pto 1	177	AGG TAG AGC TGC ATG TAA AAG T	Wang et al., 2003
OOM-lo 5.8S47B			CGC ATT ACG TAT CGC AGT TCG CAG	Schurko *et al*., 2003, Myco. Res 107: 537-544

*All primers were paired with the oomycte specific primer OOM-Lo5.8S 47 B

Reaction and cycling conditions: The PCR reaction contained: 0.2 mM dNTP, 0.4 µM each primer, 2 mM Mg^{2+}, 1U *Taq* DNA polymerase (Promega) and 10-30 ng genomic DNA in a 12.5 µL PCR reaction volume. The optimum cycling conditions, taking into consideration the differences in annealing temperatures were: 94°C 2 min; 35 cycles of 94°C for 30 sec, annealing at 63°C for 30 sec; extension at 72°C for 40 sec; followed by a final extension at 72°C for 10 minutes. However, these should be optimized for each laboratory, taking into account differences in thermocyclers, enzyme used and source of different reagents.

Electrophoresis conditions: Optimum electrophoresis condition to be able to distinguish all *Pythium* species was in 1.5% agarose gel. The small fragment size might warrant higher concentrations of agarose, and these have to be optimized for local conditions before large-scale application.

Conclusion: A multiplex PCR assay was developed to simultaneously detect and identify 6 *Pythium* species that are pathogenic to beans, and *P. oligandrum*, the biocontrol agent. We are

currently testing different methods for extraction DNA from plant tissues to assess the effect of DNA purity on PCR amplification efficiency. In addition, we are adopting different methods for extracting DNA from different soils with different clay content, so as to establish the suitability of the developed PCR assay for direct detection of these *Pythium* species in soil.

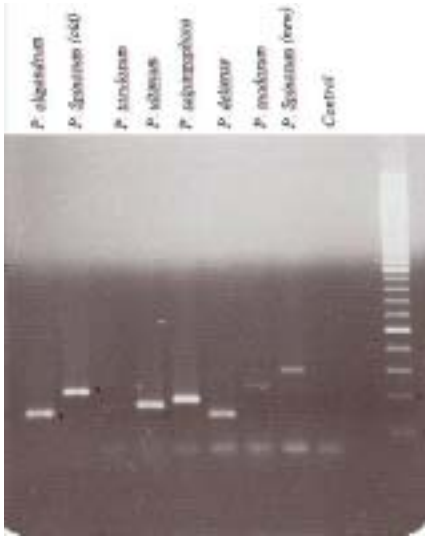


Figure 1.2.1. Detection of 6 *Pythium* species using a multiplex PCR assay. DNA from different *Pythium* species was mixed and amplified using species-specific primers that target the ITS 1 region of ribosomal DNA.

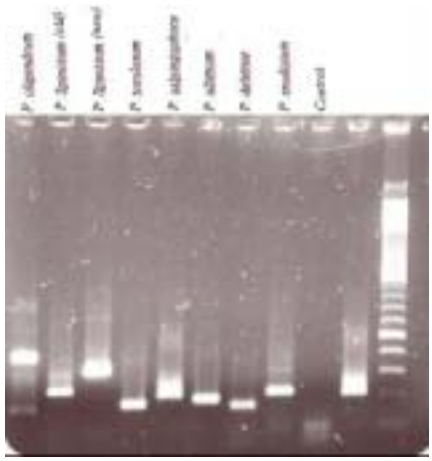


Figure 1.2.2. Suitability of multiplex PCR assay to detect different *Pythium* species. Primers specific to seven *Pythium* species were mixed, and DNA from individual *Pythium* species was added. In all cases, the desired fragment was amplified. Only with *P. oligandrum* were two fragments amplified, the desired 150 bp and a second larger fragment of approximately 400 bp.

Activity 1.2.1. Development of a molecular-based quantitative assay for *Pythium* species

Contributors: G. Mahuku and R. Buruchara

Highlight:

- We developed a competitive PCR assay for *Pythium ultimum* var *ultimum* and showed its utility to determine DNA concentration from unknown samples**.**

Rationale

The incidence and severity of root rots caused by soil borne pathogens is directly related to levels of pathogen propagules in the soil. Several management strategies are directed at reducing soil inoculum to below economic threshold levels. Although the effects of management practices can be assessed or measured on the basis of disease severity, the latter are not always the best indicators of soil pathogen population. There is therefore need to develop tools and procedures that are simple, fast and accurate for the quantification of pathogen populations directly in soils. This would facilitate development of prediction models but more importantly will facilitate determining the effects of various root rot management options on the pathogen population. It will also facilitate the understanding of factors or practices, which influence the increase of the pathogen population in the soil subsequently leading to root rots. *P. ultimum* var *ultimum* has been identified as the most important cause of bean root rots in East Africa. The objective of this project is to develop a molecular based quantitative assay, based on the competitive PCR technique that could be used to monitor propagules of *P. ultimum* var *ultimum* in the soil and to assess the effect of different management options on inoculum levels of this pathogen in the soil. It is hoped that this assay will yield information that can lead to recommendations on best practices to include in an integrated root rot management strategy.

Materials and Methods

PCR amplifcations: An isolate of *P. ultimum* obtained form diseased bean roots was used in this study. Amplification of the target *P. ultimum* DNA was done using the primers Puk1 and OOM-lo 5.8S47B previously developed by Kageyama et al., 1997, Plant Disease 81: 1155-1160; Schurko et al., 2003, Mycological Research 107: 537-544, respectively, using the following conditions: 0.5 µM of each primer, 0.2 mM dNTP mixture, 1x PCR buffer, 1.5 mM Mg^{2+}, 1U Taq DNA polymerase and 20 ng genomic DNA in a 12.5 µL PCR reaction volume. The cycling conditions were as follows: 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for I min, annealing at 55°C for 1 min and extension at 72°C for 2 min, with a final extension at 72°C for 10 min.

Creation ofa heterologous internal Standard: An internal standard was prepared by amplifying genomic DNA extracted from the soil pathogen *Macrophomina phaseolina* with primers Puk1 and OOM-lo5.8S47B (Schurko *et al*., 2003, Mycological Research 107: 537-544) at low stringency. These two primers amplify a fragment of approximately 200 bp in *P. ultimum*. Two fragments of approximately 250 and 190 bp were excised from the gel, purified and amplified at high stringency (55°C) using the same primers. After confirming the size, the fragment was cleaned and cloned into pGEM T-easy vector and maintained in transformed *Ecoli* . DNA for further studies was extracted and quantified using a scpectrophotometer and used as control DNA in developing a *P. ultimum*-specific competitive PCR assay. To determine the utility of this fragment as control DNA, varying concentrations of control DNA, ranging from 100 ng to 10 fg

were amplified either alone or in the presence of a fixed amoung of *P. ultimum* DNA, using the cycling conditions previously described.

Results and Discussion

Generation of competitor DNA fragments: The amplification of *M. phaseolina* genomic DNA under low stringency annealing conditions resulted in the production of several PCR fragments. Two fragments of 250 and 190 bp in size were excised from the gels, purified and cloned into pGEM T-easy vector. These fragments were engineered to contain the priming sites of the two *P. ultimum*-specific primers, through low stringency amplification, followed by size selection before the fragment was cloned in *Ecoli* (Figure 1.2.1.1) In competitive PCR, it is extremely important to have a competitor with a greater degree of similarity (size fragment) to the target, so as to allow for more even amplification efficiencies. In addition, the two fragments should be easily distinguished upon electrophoresis. The usefulness of the designed heterologous probe was tested by amplifying different concentrations of competitor DNA in the presence of a fixed amount of target DNA (Figure 1.2.1.2). As the concentration of competitor DNA decreases, the intensity of the amplified target DNA increases. By comparing the relative band intensities of the two fragments, a ratio is reached where the amount of target and competitor DNA are in a 1:1 ratio. The sensitivity of detection of was measured by amplifying known concentrations of the control DNA. The assay could detect down to 100 fg of competitor DNA (Figure 1.2.1.3).

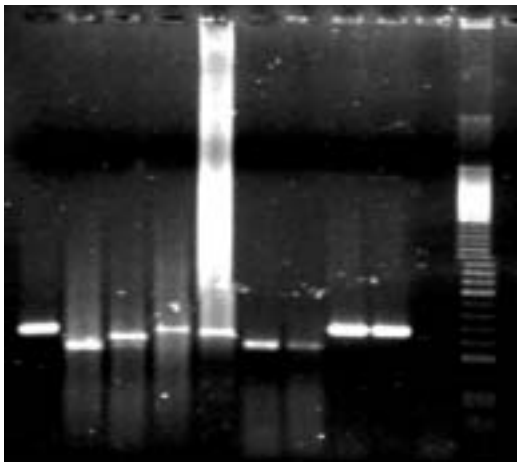


Figure 1.2.1.1. Ethidium bromide stained gel of products of candidate internal fragments for competitive PCR. Lanes 1, 8 and 9 represent *Pythium ultimum* DNA, while lanes 2 to 7 are different individual bands obtained from amplifying *Macrophomina phaseolina* DNA under low stringency conditions using *P. ultimum* specific primers Puk1 and Puk3. Fragments from lanes 2 and 3 were eventually cloned and subsequently tested for their suitability as internal controls in competitive PCR assays.

Conclusion: We successfully developed control fragment that can be used in the competitive PCR assay to estimate *P. ultimum* DNA concentration, and hence estimate pathogen inoculum levels in the soil. We are in the process of developing standard curves by amplifying DNA isolated from known concentrations of zoospores and oospores obtained from *P. ultimum* var *ultimum*. In addition, we are in the process of testing the suitability of the designed competitive PCR assay for direct estimation of *P. ultimum* propagules directly in the soil. Several procedures

for direct extraction of *P. ultimum* DNA from soils are currently being tested, by extracting known concentrations of DNA from soils differing in structure, clay and humus content. This is important, to optimize the conditions under which this assay could be applied.

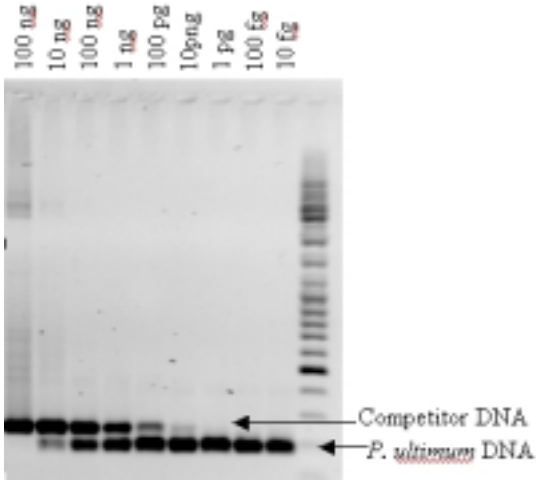


Figure 1.2.1.2. Competitive PCR products of *Pythium ultimum*. A constant unknown concentration of *P. ultimum* DNA was co amplified in the presence of competitor DNA ranging from 100 ng to 10 fg. At 1 ng concentration of competitor DNA shows an almost 1:1 ratio of control: *P. ultimum* DNA concentration.

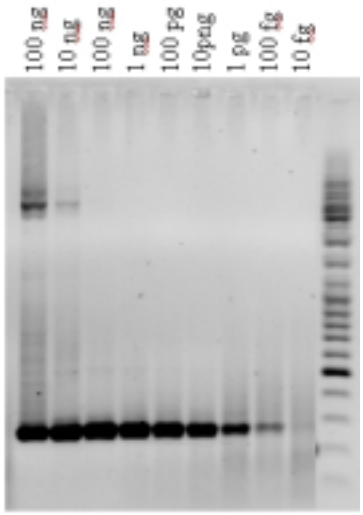


Figure 1.2.1.3 Sensitivity of the competitive PCR assay to detect target DNA

Activity 1.3. Development of molecular markers linked to the *Pythium* resistance genes in common bean genotype RWR 719

Contributors: G. Mahuku, and M. Navia, R. Buruchara (PABRA) and R. Otsyula (KARI)

Highlights:

- Three markers linked in coupling to the resistance gene in RWR 719 were identified, two were turned into SCAR markers and the potential use of these markers in MAS was demonstrated.
- This is the first report of tagging and developing a SCAR marker for a Pythium gene in common bean.

Rationale

Previous studies have shown that Pythium root rot resistance in RWR719, AND 1062 and MLB 48-89A is controlled by single dominant gene(s). Allelism tests among the resistant germplasm showed no segregation, revealing the possibility of a common locus controlling resistance to *Pythium* spp. in common bean. Field and screen house evaluations of RWR719, AND 1062 and MLB 48-89A using seven *Pythium* species confirmed the potential of this resistance locus as a source of resistance to this pathogen. This study was initiated to develop molecular markers that are linked to the resistance gene(s) so as to use marker assisted selection (MAS) to facilitate the introgression of resistance into varieties preferred by small-scale farmers in east and central Africa.

Materials and Methods

Plant material: Crosses were made between GLP2 and each of the DNA resistant parents (RWR719, AND 1062 and MLB 48-89A) to create F_2 populations. All F_2 populations were evaluated using *Pythium ultimum*, previously established as the most important and widely distributed species causing bean root rots in East and Central Africa (Mukalazi *et al*., 2001, African Crop Sci. Conference, Lagos, Nigeria). Inoculum production, and inoculation were done as described previously (Mukalazi *et al.*, 2001). Seeds of parental materials and F_2 individuals were planted in inoculated soil in wooden trays. Germinated seedlings were watered 2 times a day for three weeks to provide a favorable environment for fungal infection, establishment and development. Individual seedlings were uprooted, washed in tap water and roots scored using the CIAT 1-9 scale (Schoonhoven and Pastor-Corrales, 1987, Standard system for the evaluation of bean germplasm, CIAT). Plants with no or limited symptoms (score 1-3) were rated as resistant, and the rest of the plants as susceptible.

DNA extraction and Marker identification: Young trifoliate leaves were collected from the resistant and susceptible parents and resistant and susceptible F_2 progenies, and DNA was extracted using the procedure described by Mahuku (Mahuku *et al*., 2004, Plant Molecular Biology Reporter 22: 71-81). Five resistant and 5 susceptible plants, including the parents were used to evaluate 300 RAPD and 50 RAMS primers as previously described (CIAT, Bean Project Annual Report 2004). Candidate markers showing evidence of correlation to disease resistance or susceptibility were further evaluated on an additional 10 resistant and susceptible F_2 plants. Where polymorphism was maintained, the potential markers were evaluated on the entire F_2 population (Table 1.3.1). The marker scoring data in the F_2 were merged with the disease scoring data for linkage analysis using the computer program MAPMAKER (Lander *et al.,* 1987, Genomics 1: 174-181).

Table 1.3.1. Response of common bean genotypes to inoculation with three-root rot causing *Pythium* species.

Bean genotype	Reaction to 6 *Pythium* species [a]	Candidate markers		
		Scar-PAA19	Scar-BA08	(GT)n
A 240	1	+[b]	+	+
MLB-49-89A	1	+	+	+
MLB-40-89A	1	+	+	-
RWR 719	1	+	+	+
Scam80-cm/15	1	+	+	+
AND 1064	1	+	+	+
AND 1062	1	+	+	+
RWR 1091	1	+	+	+
GLP 585	9	-	-	-
Scam- KWD	9	-	-	-
GLP2	9	-	-	-
URUGEZI	9	-	-	-
CAL 96	9	-	-	-

[a]The genotypes were evaluated with 6 *Pythium* species; *P. ultimum*, *P. salpingophorum,P. spinosum,P. tolorosum,P. chamaehyphom* and *P. pachycaule*. The reactions are based on the CIAT 1-9 scale and different scores for the same genotype represence different scores from different Pythium species.
[b] + represent presence of a marker and – absence of a marker.

Establishing the suitability ofcandidate marker for MAS: Candidate markers were evaluated on nine bean genotypes (RWR 719, MLB 49-89A, AND 1062, A 240, SCAM 80-CM/15, MEX 54, CAL 96, Urugezi and GLP 2) that are either resistant or susceptible to Pythium root rots under greenhouse and field conditions (Table 1.3.1)

Development ofSCAR markers: Candidate fragments were excised from agarose gels, cloned and sequenced as described by Mahuku *et al.* (2004, Crop Science 44: 1817-1824). Primers were designed using the Primer3 software (Center for Genome Research, Whitehead Institute, MA, USA - http://www-genome.wi.mit.edu/cgi-bin/primer/primer3). Developed primers were used to amplify DNA from parental materials, and two resistant and susceptible F_2 individuals. If polymorphism was maintained, the designed SCAR primers were tested in ten resistant and ten susceptible individuals. In the case of an identical sequence length, the fragment from the susceptible individuals was cloned and sequenced. The sequences derived from resistant and susceptible individuals were then aligned using the program MEGALIGN within DNAStar, and where possible the primer pairs were re-designed to exploit differences between the resistant and susceptible sequences.

Results and Discussion

Marker identifcation: Of the 300 RAPD and 50 RAMS primers evaluated, four RAPD primers, (OPAA19, OPY20, OPG3 and OPBA08) and one RAMS marker $(VHVGT)_5G$) segregated in coupling phase with the resistance gene in RWR 719 (Figure 1.3.1 A, B, and C), three RAPD primers (OPY20, OPF3 and OPG3) segregated in coupling phase with the resistance gene in MLB 49-89A. No marker was observed for the gene in AND1062. Further testing of RWR 719 potential markers on 10 resistant and susceptible individuals confirmed the potential of OPAA19, OPBA08 and (GT) n as molecular markers linked to the RWR 719 resistance gene. Since RWR 719 is currently being used in programs to introgress resistance to pythium root rot into commercial varieties in Eastern Africa, we focused our marker development efforts on this

genotype. Linkage analysis after testing the marker on 150 F_2 individual plants showed that the OPAA19 marker was located at 1.5 cM, the OPBA08 at 4 .0 cM and (GT)n marker at 6.3 cM from the resistance gene.

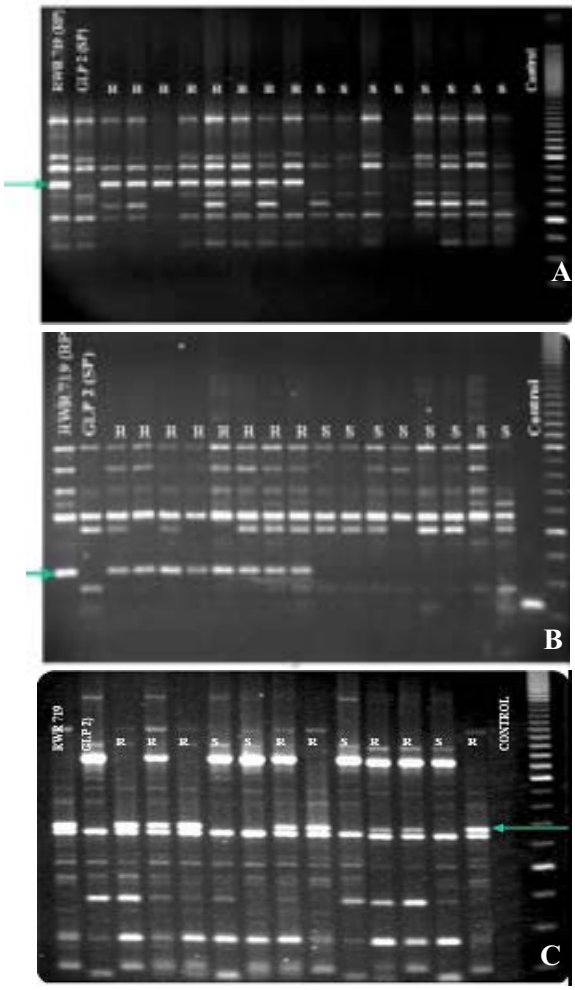


Figure 1.3.1. RAPD (A) OPAA19, (B) OPBA08, and RAMS GT $(VHVGT)_5G$) linked in coupling to the pythium resistant gene in the common bean genotype, RWR 719.

Validation of markers for MAS: Amplification of parental materials and selected susceptible and resistant bean genotypes revealed the potential of all three markers for marker assisted selection (MAS) breeding. The fragments associated with resistance were present in all resistant and absent from susceptible genotypes (Figure 1.3.2A and B), raising the possibility that the resistant genotypes tested in this study might carry the same resistance gene locus, with the same or different alleles for conditioning pythium root rot resistance. These results concur with the allelism test, where no segregation was observed in resistant x resistant crosses involving RWR 719, AND1062, SCAM 80/15 and MLB49-89A. Furthermore, all cultivars tested (RWR 719, AND1062, SCAM 80/15 and MLB49-89A) have A240 in their pedigree. Greenhouse evaluations of A240 showed that it was resistant to *P. ultimum* var. *ultimum*, and the fragment associated with resistance for all potential markers was present in A240. It is likely that this genotype is the origin

of resistance to pythium root rots. We are currently evaluating other genotypes that have A240 as one of the parents to test this hypothesis.

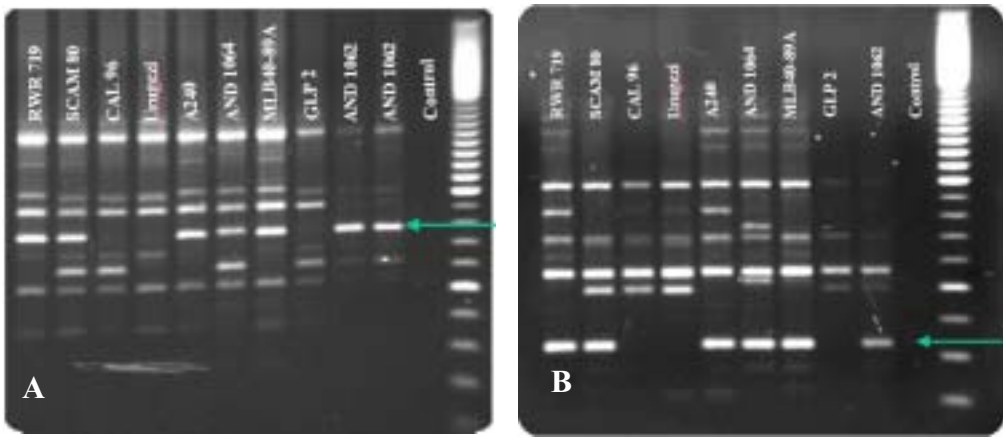


Figure 1.3.2. Validation of molecular markers outside the mapping population: (A) OPAA19 and (B) OPBA08. The fragments associated with resistance are present in all resistant and absent from susceptible genotypes.

SCAR Marker: Three sequence characterized amplified region (SCAR) markers were developed for Of the three SCAR markers developed OPAA19, OPBA08 and (GT) n markers. The SCAR marker derived from OPAA19 was polymorphic and co-dominant (Figure 1.3.3). The SCAR marker derived from BA08 was polymorphic and dominant, while the $(GT)_n$ derived marker amplified a similar sized fragment from susceptible and resistant plants. PCR products from resistant and susceptible parents amplified using these primers were cloned and sequenced. Alignment of resistant and susceptible plant sequences revealed polymporphisms and new primers targeting these differences were designed and are currently being synthesized. In addition, RILs have been developed from the RWR 719 x GLP 2 cross and these will aid further identification and development of other markers, and increase the efficiency of marker use in MAS.

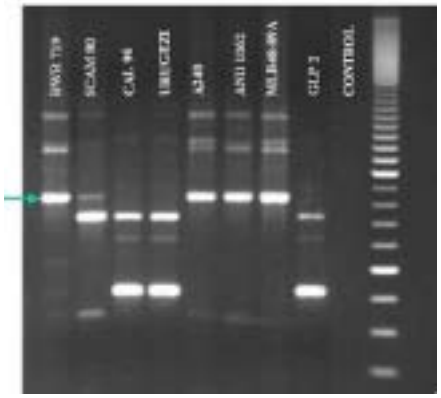


Figure 1.3.3. Amplification of resistant and susceptible bean genotypes using the SCAR primers derived from the OPAA19. The fragment associated with resistance was present in resistant and absent from susceptible genotypes.

Conclusions: Three markers linked in coupling to the resistance gene in RWR 719 were identified, and the potential of these markers in MAS was established. SCAR markers derived from the OPBA08and OPAA19 RAPD primers were developed and their potential utility for MAS demonstrated. We are in the process of validating these SCAR markers outside the mapping population. In addition, we are currently in the process of developing SCAR markers for the VHVGT)$_5$G markers, as well as evaluating RGAs and bean microsatellites so as to identify more markers and saturate the region. This is important as it will increase the utility of these markers in MAS.

Activity 1.4. Characterizing and monitoring pathogen and insect diversity

Contributors: R. Buruchara, V. Gichuru, (graduate student), S. Buah, C. Acam and S. Musoke (CIAT), F. Opio and M.A. Ugen (NARO)

Highlights:

- Three major intercrops of beans i.e. maize, sorghum and peas, in the bean-based system of southwestern Uganda were affected by root rots implying that they may be hosts of the pathogens.
- Management options effective for bean root rots are also beneficial to other crops such as sorghum and field peas in bean based cropping system. Formulating management strategies for root rots need to consider a systems approach rather than a crop's approach.
- Several Pythium spp were recovered from crops grown in association with beans. *P. ultimum* is the most frequent.
- Mortierella (MS10) has was shown been observed to have antagonist effects to pathogenic Pythium isolates with marked reduction in disease severity in screenhouse studies offering potential as a biocontrol agent against Pythium root rot.

Activity 1.4.1. Characterization and distribution of *Pythium spp*. associated with other crops in a bean based cropping system in southwestern Uganda

Rationale

Mukalazi (2002, PhD dissertation, Makerere University, Kampala, Uganda, pp 59-60) identified eleven *Pythium* species from Kabale district associated with bean root rot in Uganda, five of which were pathogenic on beans under field and screenhouse conditions. He also observed wide diversity of *Pythium spp* based on morphological, cultural and molecular characteristics. *Pythium* is an oomycete with a wide host range, which renders it a highly likely source of increased epidemics. There is very limited information on the role of various cropping systems on *Pythium* diversity and their role in root rot epidemics of beans in East Africa. Advances in plant and molecular pathology in particular provide a host of tools, which could be used to elucidate the diversity of *Pythium* spp under different cropping systems. We carried out studies to investigate species of *Pythium* associated with the crops grown as intercrops or in rotation in the bean based systems in southwestern Uganda.

Materials and Methods

Eighty *Pythium* isolates were obtained from intercrops of beans in Kabale district. DNA was extracted from harvested mycelia according to the procedure described by Mahuku (2004, Plant Molecular Biology Reporter 22:71-81). PCR analysis was performed using Oomycete ITS region primers to differentiate *Pythium* from other closely related fungi. The amplified products were purified and sequenced. The resultant sequences were edited for accurate peak scorings and later subjected to a nucleotide-nucleotide blast search at National Center for Biotechnology Information (NCBI) website. The sequences were compared to 10 most relevant matches in the GenBank and the best match was taken as the species of the isolate.

Results and Discussion

Out of the 80 isolates characterized, 19 species were identified (Table 1.4.1.1). Five of these have been reported in our previous pathogen characterization studies in Uganda but fourteen were new additions. Thirty percent of the isolates were *Pythium ultimum* followed by *P. acanthicum* (12.5%), *P. spinosum* (11.25%) and *P. torulosum* (7.5%). *P. arrhenomanes, P. macrosporum*, *P. mamilatum, P. orthogonon*, *P. conidiophorum, P. erinaceum, P. periplocum* and *P. vexans* were the least prevalent, each of which occurred only once. Species distribution map is shown (Figure 1.4.1.1). These results are consistent with past observations that overall *P. ultimum* is the most frequent species in the region in the case of beans and their intercrops. These results show that other crops in the bean system play a role as likely hosts to bean pathogenic *Pythium spp*. It also indicates that other crops are likely hosts to *Pythium spp*, which had not been previously isolated from, beans. Further studies will determine the pathogenicity of *Pythium spp*. from other crops on beans to establish if other crops in the bean system play a role as likely hosts to bean pathogenic *Pythium spp*. The knowledge generated from this study is crucial to formulating an effective management strategy for bean root rot in similar cropping systems.

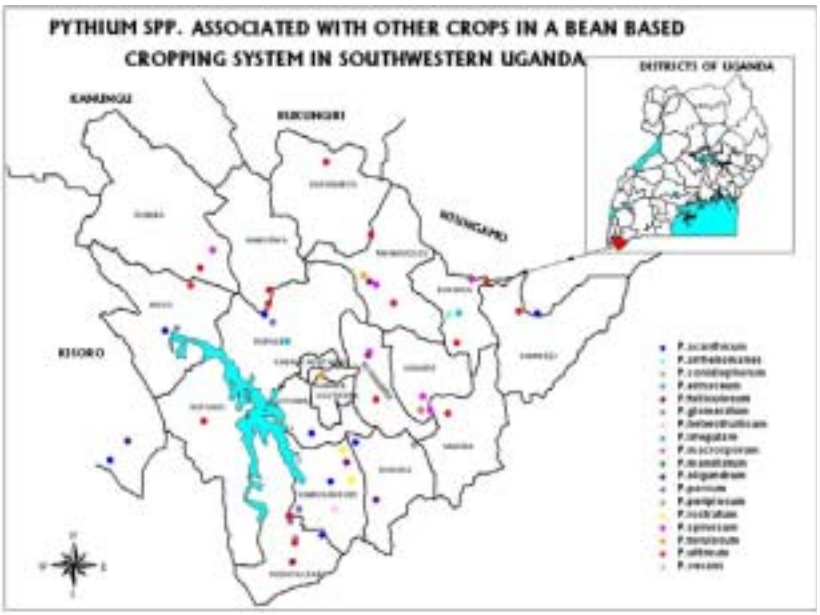


Figure 1.4.1.1. A distribution map of *Pythium* species identified and found to be associated with other crops in a bean based cropping system in southwestern Uganda

Table 1.4.1.1. Identification by sequencing of *Pythium* isolates from other crops in a bean based cropping system in southwestern Uganda

	Major Crops sampled												
***Pythium* spp.**	**Irish**	**Sorghum**	**Maize**	**Bean**	**Sweet potato**	**Cabbage**	**Peas**	**Tomatoes**	**Millet**	**Wheat**	**Bananas**	**Weed**	**Total**
P. ultimum	8	6	4	-	1	2	1	-	-	1	1	-	24
P. acanthicum	5	3	-	1	-	-	-	-	-	-	-	1	10
P. spinosum	3	4	-	-	-	-	1	1	-	-	-	-	9
P. torulosum	2	-	2	1	-	-	1	-	-	-	-	-	6
P. folliculosum	1	1	1	2	-	-	-	-	-	-	-	-	5
P. oligandrum	1	2	2	-	-	-	-	-	-	-	-	-	5
P. parvum	-	1	-	-	-	2	-	-	1	-	-	-	4
P. irregulare	2	-	-	-	-	-	-	1	-	-	-	-	3
P. glomeratum	2	-	-	-	-	-	-	-	-	-	-	-	2
P. heterothallicum	-	-	-	-	2	-	-	-	-	-	-	-	2
P. rostratum	1	-	-	-	1	-	-	-	-	-	-	-	2
P. arrhenomanes	-	1	-	-	-	-	-	-	-	-	-	-	1
P. macrosporum	1	-	-	-	-	-	-	-	-	-	-	-	1
P. mamilatum	1	-	-	-	-	-	-	-	-	-	-	-	1
P. orthogonon	-	-	-	1	-	-	-	-	-	-	-	-	1
P. conodiophorum	-	-	-	1	-	-	-	-	-	-	-	-	1
P. erinaceum	-	1	-	-	-	-	-	-	-	-	-	-	1
P. periplocum	-	-	-	-	1	-	-	-	-	-	-	-	1
P. vexans	-	1	-	-	-	-	-	-	-	-	-	-	1
Total	**27**	**20**	**9**	**6**	**5**	**4**	**3**	**2**	**1**	**1**	**1**	**1**	**80**

Activity 1.4.2. Pathogenicity of *Pythium* spp on beans, Kawanda, screen house

Rationale

The genus *Pythium* consists of more than 100 species with unique ecological preferences and includes important plant pathogens, parasites of other soil or water organisms, and saprophytes that survive on dead organic matter. Close to 30 different *Pythium* species have been encountered in the East African region. However, not all of the identified species have been checked for pathogenicity on important crops of the region. Therefore, the objective of this study was to test the pathogenicity of some isolates associated with beans. The knowledge obtained will help in designing management strategies for the disease in the region.

Materials and methods

Pythium isolates stored on PDA slants at 4°C were reactivated and sub-cultured at 24 C. Inoculum of each isolate was raised independently on millet grains for 12 days and incorporated in sterilized soil at a ratio of 1:8 v/v inoculum to soil. Three susceptible bean varieties (CAL 96, Kanyebwa, and K20) and one resistant variety (RWR 719) were grown on infected soil Disease evaluation was done 3 weeks after planting using the 1-9 CIAT scale. Isolates with mean disease score of 5.0 on CAL 96 were considered to be pathogenic to beans.

Results and Discussion

Of the 6 isolates tested two were found to be non-pathogenic (*P. diclinum*), whereas four were pathogenic (Table 1.4.2.1).

Table 1.4.2.1. Pathogenicity of different *Pythium* species on bean cultivars, Kawanda.

		Bean cultivars			
Isolate	***Pythium* spp.**	**CAL 96**	**Kanyebwa**	**RWR 719**	**K20**
GS1	*P. diclinum*	3.15	2.20	1.24	2.11
GS4	*P. diclinum*	3.26	3.22	1.65	3.65
KB15A	*P. rostratum*	6.41	6.29	2.38	6.34
KIS8B	*P. irregulare*	5.07	4.23	1.23	3.80
MC2C	*P. torulosum*	5.97	4.57	1.66	3.55
MR11a	*P. vexans*	7.46	7.57	1.58	6.96
MS61 (+ve)	*P. ultimum*	8.53	8.57	1.90	8.49
-ve control	No *Pyth*_spp	1.00	1.03	1.00	1.00

Note: Disease scale (1-9) 1 = no root symptoms; 3 = 10% of the hypocotyl and root tissues have lesions; 5= 25% of the hypocotyl and root tissues lesions 7= 50% the hypocotyl and root tissues have lesions and the root system suffers a considerable decay; 9= 75% or more of the hypocotyl and root tissues have lesions and the root system suffers advanced stages of decay and considerable reduction (Abawi and Pastor Corrales, 1990, CIAT, Cali, Colombia, 114 pp).

These results from four plantings clearly showed that *P. ultimum* was the most pathogenic species followed by *P. vexans*, *P. rostratum*, *P. torulosum*, and *P. irregulare* in that order. These results are consistent with pathogenicity tests done by Mukalazi (2000, PhD thesis, Makerere University, Uganda) in which he recorded disease scores 6-9 for *P. ultimum* and *P. torulosum* for several isolates. There is no previous pathogenicity data on *P. diclinum*, *P. rostratum*, *P. irregulare*, and *P. vexans* from this region.

Activity 1.4.3. Characterization of bean leaf crumple virus

Contributor: F. Morales

Bean leaf crumple virus continues to affect snap beans in the Cauca Valley of Colombia (Figure 1.4.3.1). All of the available snap bean lines evaluated to date (over 120 lines) and some backcrosses made to red-seeded genotypes from Honduras, have been severely affected by this whitefly-transmitted virus. It is necessary to undertake a serious breeding for resistance project for the predominant snap bean variety grown in the Cauca Valley ('Blue Lake'), using the black-seeded Mesoamerican sources of resistance identified by Bean Virology. This virus has been occasionally observed to attack bush beans under experimental field conditions. Recently, a line identified as SUG 131, showed a 100% disease incidence due to this virus under field conditions at CIAT.

Figure 1.4.3.1. Broad pathogenicity range of a new whitefly-borne virus in common bean genotypes under field conditions in the Cauca Valle, Colombia.

Activity 1.4.4. Monitoring Peanut stripe virus in common bean

Peanut stripe virus (PStV) is currently considered a strain of BCMV, but this virus has a distinct biological (pathological) behavior (CIAT, 2002, Bean Project Annual Report). In 2005, reports of a new viral disease affecting common bean in Mexico, was brought to our attention. In serological assays, the causal virus was shown to be a member of the *Potyvirus* genus. The virus was amplified using potyvirus-specific primers and then cloned for partial sequencing. The sequence obtained corresponded to Peanut stripe virus, indicating that this virus is being disseminated in Latin America by aphids and probably through sexual seed of common bean and other legumes, including peanut. The Peanut stripe strain of BCMV from CIAT (Figure 1.4.4.1**)** and Mexico show capsid protein aminoacid homologies > 95% (Figure 1.4.4.2). Peanut stripe has a pathogenicity range in common bean similar to the New York 15 strain of BCMV, but differs in certain groups of differential cultivars. For instance, NY 15 infects Michelite 62 and Red Mexican 34, whereas PStV does not; but PStV infects Great Northern 123, not attacked by NY 15. PStV does not induce systemic necrosis in the BCMV differential genotypes, and there are several sources of resistance to this virus that could be used, should the need arise.

Figure 1.4.4.1. Symptoms induced by *Peanut stripe mosaic virus* (currently *Bean common mosaic virus*) in a susceptible common bean cultivar.

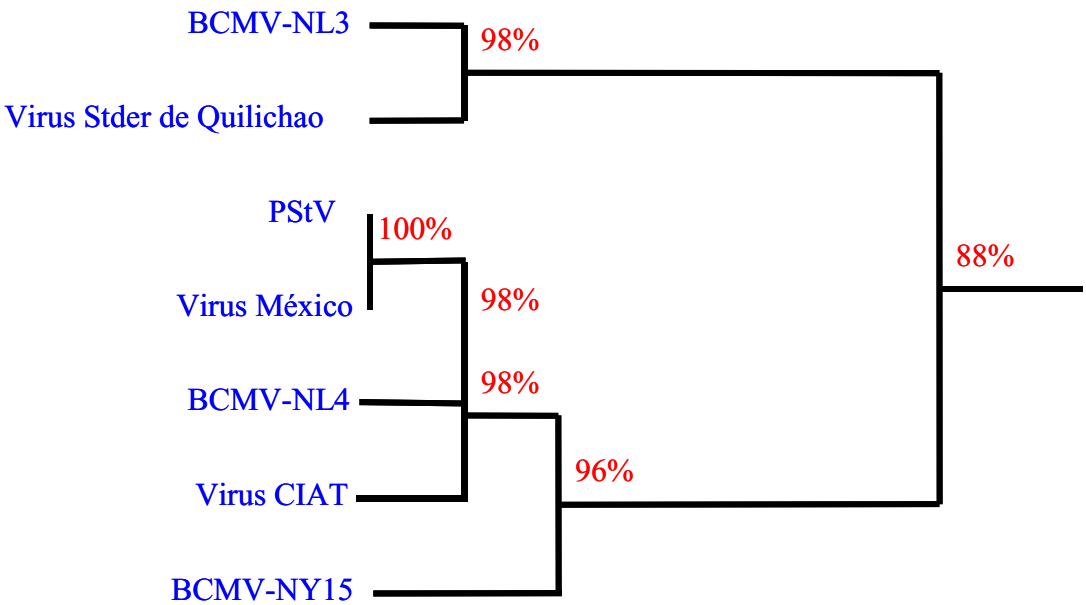


Figure 1.4.4.2. Phylogenetic Tree. Multiple alignment of the aminoacid sequence of the 3' of the Coat protein gene of BCMNV NL3, and BCMV NL4 and NY15; *Peanut stripe virus* (PStV) and bean viruses detected in Santander de Quilichao, CIAT and México (DNAMAN versión 4.13).

Activity 1.4.5. Characterization of a virus inducing systemic necrosis in common beans planted in Santander de Quilichao, Cauca, Colombia.

The potyvirus isolated from common bean in Santander de Quilichao, Cauca, in 2004, has been shown to be similar in the genomic composition of their capsid protein genes, but quite distinct from the necrosis-inducing BCMNV-NL3 strain in their host range. The pathogenicity spectrum of this legume potyvirus isrestricted to cultivars Widusa and Black Turtle Soup (Group 8), whereas BCMNV-NL3 is known to cause systemic necrosis in *I* gene groups 8, 9a and 9b.The virus also differs from BCMNV-NL3 in its pathogenicity to strain-specific recessive genes. The SQ potyvirus is particularly virulent in Groups 1 and 4, and does not induce systemic infection in various cultivars shown to be susceptible to BCMNV NL3. The phylogenetic position of this new virus is shown in Figure 1.4.4.2.

Activity 1.4.6. Detection and characterization of viruses affecting common bean in northwestern Argentina

Argentina is the main exporter of black-seeded beans in the world thanks to the improved cultivars developed by CIAT. This technical assistance has been provided in view of the high yields and low production costs that characterize bean production in northwestern Argentina (NWA), which makes black-seeded beans available to developing countries at highly reduced prices. In 2005, a virus survey was conducted in NWA to monitor the phytosanitary situation of the crop. The results of this survey showed that *Bean golden mosaic virus* is still the predominant virus in the region, and that this virus has not changed significantly in the past decade. The 'achaparramiento' (bean dwarf mosaic) disease of common bean that greatly affected common bean production in NWA in the late 1970s and early 1980s, is still present but only affects common bean genotypes that have not been improved for their resistance to these viruses (Figure 1.4.6.1). Whereas genetic recombination between the causal agents of bean dwarf mosaic and bean golden mosaic has been detected in some samples, the genetic resistance deployed in NWA is effective against both viruses.

Figure 1.4.6.1. Bean dwarf mosaic-affected field in N. W. Argentina, showing that this virus is still present in this region. All commercial cultivars deployed by CIAT in this region since 1980 are resistant to this virus.

Because these virus problems are associated with the presence of their whitefly vector, *Bemisia tabaci* , a study on the distribution of biotypes in NWA was carried out. Interestingly, the original A biotype was

still present in all the samples assayed, but there are reports on the presence of the new B biotype in NWA.

The high populations of *Btabaci* in NWA, however, pose a constant threat to common bean production, not only as vectors of begomoviruses, such as bean golden mosaic and bean dwarf mosaic viruses, but as vectors of other genera of viruses as well. In the survey conducted, a carlavirus (*Cowpea mild mottle virus*), was detected in some samples. Fortunately, this virus transmitted by *Btabaci*, does not affect bean production significantly. In Brazil, where this virus probably comes from, it only moderately affects 'Jalo' and 'Manteiga' common bean genotypes. In NWA, virus symptoms are so mild that they are usually overlooked (Figure 1.4.6.2).

Another begomovirus found to affect common bean in NWA in 1995 (CIAT, Bean Project Annual Report 1995), and later on reported as a pathogen of tomato in Brazil, appears to be Potato deforming mosaic virus, originally found in 1981 affecting potato in the province of Buenos Aires, Argentina. This virus seems to affect common bean as well in Brazil and Argentina.

Figure 1.4.6.2. Symptoms induced by *Cowpea mild mottle virus* in common bean, a filamentous virus transmitted by the whitefly *Bemisia tabaci*.

Activity 1.5. Identification of a virus associated with cassava plants affected by frogskin disease

Contributors: L.A. Calvert, M. Cuervo, I. Lozano, N. Villareal and J. Arroyave.

Rationale

The causal agent of cassava frogskin disease (CFSD) has remained unknown since the disorder was first identified in 1971. Although a viral agent has been suspected, it has been difficult to determine the etiology of CFSD. Virus-like particles of 70 and 45 nm in diameter were found in affected cassava plants and partially purified preparations, respectively. Nine species of dsRNA were associated with this disease. Eight cDNA clones were synthesized from the dsRNAs extractions. The putative proteins predicted from the sequence of the cassava virus cDNA clones have similarity with the P1, P3, P4, P5, P10 proteins of rice ragged stunt virus. Hybridization analyses of the dsRNA extractions identified the S1, S3, S4, S5 and S10 genomic segments in the CFSD affected plants, but not in healthy controls. Blind testing of diverse isolates identified most but not all the positive samples. No false positives were detected.

Introduction

Cassava frogskin disease (CFSD) is a disorder of unknown etiology that affects cassava and was first reported in 1971 from southern Colombia (Kimura *et al.*, 2001, Biosci. Biotechnol. Biochem. 65: 125-1283), and is endemic in the Amazon regions of Colombia, Peru, and Brazil. In the Amazon region, one common name for CFSD is "Jacaré" (caiman). The disorder is also present in Venezuela and Costa Rica. While the principal means of transmission are infected stem cuttings that are used to propagate cassava, there is circumstantial evidence suggesting that an aerial vector spreads disorder. The disease is transmitted through grafting (Upadhyaya *et al*., 1998, Arch. Virol. 143: 1815-1822).

In a typical CFSD affected cassava plant, there are no leaf symptoms, but the base of the stem can be swollen and the root periderm and corky layers enlarge to form raised lip shaped fissures (Fukushi *et al*., 1962, Virology 18: 192-198). Roots that are severely affected do not fill with starch. In areas where the disorder is prevalent, the yield losses can be 100%. In a limited number of cassava landraces, CFSD affected plants are stunted and the leaves develop mosaic symptoms. The roots of these varieties are either stunted or have root fissures typically associated with CFSD. When the temperatures are cooler, the symptoms tend to be more severe. When the temperature is kept constant at 30^{o}C, there is no symptom development in those landraces that normally express foliar mosaic symptoms (Fukushi *et al*., 1962, Virology 18: 192-198). CFSD can be eliminated from an affected plant by heat treatment and meristem tip culture (Milne & Lovisolo, 1977, Advances in Virus Research 21: 267-341). *In vitro* shipment of disease free certified plants is recommended for the safe movement of cassava germplasm (Calvert, 1994, International Crop Network Series Report 10: 163-165).

This report includes evidence on dsRNA genomic segments and cDNA clones that were associated with CFSD affected plants. To date about 5000 bases or 20% of the genome of CFSV has been sequenced.

Materials and Methods

Source of host plants and isolates: The CFSD affected plant materials were collected in the Andean and Amazonian regions of Colombia and maintained in greenhouses by vegetative propagation. The CFSD isolates used in this study were obtained from the cassava clones designed as Secundina 5, Secundina 80, Valluna 29, CM-5460-10, SM 909-25, Regional Tolima, CMC40, Amazonas 16, and Catumare Jamundi.

The healthy control plants were obtained from CFSD-free materials that were subjected to heat therapy and cultured *in vitro*. The *in vitro* plants were hardened and subsequently maintained in a greenhouse. When Secundina (CIAT accession CM 6014) is affected with CFSD, it develops foliar mosaic symptoms. Control test plants were periodically grafted to Secundina to assure that they had not become affected with CFSD.

Double-stranded RNA extraction and análisis: A modification of the method of Morris and Dodds (1979, Phytopathology 69: 854-858) was used to extract double-stranded RNA (dsRNA) from CMD or FSD affected plants. Young leaves or petioles were frozen with liquid nitrogen and homogenized with two volumes of extraction buffer (2X STE, 10% SDS, 1% bentonite, and 0.5% 2-mercaptoethanol) and 0.5 volumes of chloroform:pentanol (24:1). The extracts were centrifuged for 10 min at 8,000 *g*, and the aqueous phase was collected. Ethanol was added to a final volume of 16.5%, and for each gram of tissue, 0.1 g CF-11 cellulose was added to the extracts. The slurries were stirred for 30-60 min and poured into columns and drained. The columns were rinsed with 100 ml of 1X STE containing 16.5% ethanol. The columns were rinsed with 0.1 ml of 1X STE, and the ds-RNAs were eluted using three 0.1 ml aliquots of 1X STE. The nucleic acids were precipitated with 2.5 volumes of absolute ethanol followed by centrifugation. The pellets were dried and resuspended in 1X TAE buffer (0.04M Tris acetate, 1 mM

EDTA adjusted to pH 7.8 with acetic acid) containing 5% glycerol, bromo-phenol blue and xylene cyanol. The preparations were run on a 5% polyacrylamide or 1% agarose gels using a continuous buffer system of 1X TAE. On most gels, the 1-kilobase ladder from BRL (Bethesda, MD) was used as the marker. To more accurately estimate the M_r of the dsRNA species, a series of polyacrylamide gels were run using Bozarth's dsRNA standard markers (Bozarth & Harley, 1976, Biochim. Biophys. Acta 432: 329-335). The gels were stained with ethidium bromide (1 εg/ml) and examined using a UV-transilluminator.

In order to test the type of nucleic acid, the samples were subjected to digestion using a concentration of 0.1 U/µl RQ-1 Dnase (Promega), 1.2 U/µl S1 nuclease, or 0.9 εg/ml Rnase A in the presence of either high (2X) or low (0.1 X) SSC (0.15M NaCl, 0.015 M sodium citrate, pH 7.0). The samples were analyzed using TAE polyacrylamide electrophoresis.

In some cases, the dsRNAs were purified from 2% low melting point agarose gels. After staining with ethidium bromide, selected gel pieces were melted a 70 ºC, 2X STE, 2% SDS and 0.1% bentonite were added, and the extraction procedure describe for the plant material was followed.

The synthesis of cDNA from the dsRNA: The syntheses of cDNAs were started by denaturing 5 σg dsRNA and 500 ng of random (hexameros) (Gibco BRL) or 10 bases oligonucleótidos (Operon) primers using 40 Mm methyl-mercuric hydroxide at a final volume of 13 σl. The mixtures were incubated for 10 min at room temperature and frozen using liquid nitrogen. The samples were the allowed to thaw out and immediately processed. The first strand was synthesized in a final volume of 40 σl containing 50Mm Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each dATP, dCTP, dGTP, dTTP, 40 U of Rnase in (Promega) and 400 U of SuperScript II RT (Gibco, BRL). The mixture was incubated for 60 min a 37∀C. Then an additional 200 U of SuperScript II RT was added to the mixture and the reaction was allowed to continue for 30 min. The reactions were subjected to 70∀C for 1 min and placed in ice water for 2 min. To the 40σl of the first strand reaction 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.15 mM η-NAD^+, 0.25 mM each dATP, dCTP, dGTP, dTTP, 1.2 mM DTT, 25 U *Ecoli* Ligase, 40 U *Ecoli* Polymerase, 4 U *Ecoli* RNase H were added and the final volume was 150σl. After the mixture was incubated for 3 h at 16∀C, 30 U of T4 DNA Polymerase was added and the reaction was continued at 16∀C for 10 min. The reaction was stopped by the addition of 10 σl of 0.5 M EDTA, pH 8.0 and treated with phenol:chloroform:isoamyl alcohol (25:24:1). The cDNAs were precipitated with 1/10 volume of 7 M ammonium acetate and 2.5 volumes of 95% ethanol and resuspended in sterile DEPC treated water. The cDNAs were modified by adding a 3'A-overhangs and ligated in to the pCR 2.1 vector (TA cloning Kit, Invitrogen).

Elution and amplification of selected cDNA: The polymorphic cDNA amplified products were eluted from pieces of the agarose or polyacrylamide gels. The selected pieces of the polyacrylamide gels were soaked in water for 10 min at room temperature then an additional 15 min at 65∀C. The aqueous portion containing the cDNA was subject to an ethanol precipitation and the pellet was resuspended in sterile water. Five σl of each sample was amplified using 10 mM dNTPS, 1X PCR buffer, 2 mM $MgCl_2$, 0.2 σl de 10 U/σl Taq polymerase final volume of 20 σl. The PCR profiles were the same used in the original amplification, and the products were visualized used agarose gel electrophoresis.

Cloning and sequencing of PCR products: The PCR products were cloned into the TA plasmid (Invitrogen). Plasmid DNA was purified using Wizard™ plasmid purification columns (Promega). Nucleotide sequences were determined using an ABI Prism 377 sequencer (Perkin-Elmer) by the dideoxynucleotide chain termination procedure (Shikata *et al*., 1979, Ann. Phytopathol. Soc. of Jpn. 458: 436-443) using the ABI dye terminator reaction ready kit. The sequence data were analyzed using SEQUENCHER version 4.1.2 for Macintosh, NCBI BLASTX, and DNAMAN Version 4.13 (Lynnon Biosoft, Vaudreuil, Quebec).

Results and Discussion

Double-stranded RNA extraction and analysis: Nine species of dsRNA were visualized in plants affected with CFSD, but not in the healthy controls. The nine species of nucleic acids present in the PAGE gels were determined to be dsRNA by digestion experiments using RQ1 DNase, RNase A, and S1 nuclease. These nine species of nucleic acids were not digested by RQ1 DNase or S1 nuclease; were resistant to digestion by RNase A in high salt, but were digested in low salt. It was determined that these were ds-RNAs and their size was estimated using ds-RNA markers. The sizes of the nine species were estimated to be between 4000 to 1000 bases (Table 1.5.1).

Complementary DNA cloning of the dsRNA associated with CFSD: The cDNA clone designed CFSV-S1 and was 228 nucleotides in length and contained an open reading frame of 62 amino acids. The putative protein had 39% identity and 55% similarity with the homologous region of the RRSV P1 protein (Figure 1.5.1). The clone CFSV-S1 was used to localize the S1 genomic segment in agarose gels. The dsRNA genomic segments in this region were eluted from the agarose gels and cDNA clones were obtained. One cDNA clone was 867 nucleotides in length and contained an open reading frame of 288 amino acids. When it was determined that this cDNA clone had similarity with the RNA dependent RNA polymerase protein encoded by the genomic segment 4 of RRSV, and it was designated as CFSV-S4-867. A second cDNA clone was 580 nucleotides in length and contained an open reading frame of 159 amino acids. It had 367 nucleotides in common with CFSV-S4-867 and the contig of these clones were designated as CFSV-S4-1 and were submitted as one accession. The putative protein based on the contig of the cDNA clones was 355 amino acids and had 37% identity and 55% similarity with RRSV P4 (Figure 1.5.2). A third cDNA clone with similarity to RRSV RNA 4 was identified (Figure 1.5.1) and designated CFSV-S4-2. A comparison of the protein encoded by RRSV RNA 4 (Upadhyaya *et al*., 1997, Arch Virol. 142:1719-26) and the putative protein derived from these three CFSV cDNA clones is shown in Figure 1.5.3. Two additional cDNA clones were identified to that have similarity with RRSV RNA 3 (Figure 1.5.1) and one was designed as CFSV-S3-1 (GenBank accession DQ139868) and the other as CFSV-S3-2. Another cDNA clone was identified as having similarity with the RRSV RNA 10 (Vos *et al*., 1995, Nucleic Acids Res. 23: 4407-4414) and was designated at CFSV-S10 (Figure 1.5.1).

Association of cDNA clones with CFSD: Double-stranded RNA was extracted from individual healthy and CFSD affected plants and transferred to membranes. The CFSV-S5-351 clone hybridized to a species of dsRNA of approximately 3000 nucleotides that was extracted from CFSD affected plants. This genomic segment was detected in all nine isolates of CFSD that were tested, and was absent in the healthy controls. Both the CFSV-S1 and the CFSV-S4 clones hybridized to species of dsRNA that were approximately 4000 nucleotides that were extracted from CFSD affected plants. The genomic segment 4 was detected in all nine isolates of CFSD that were tested, but the S1 hybridized readily with Valluna 29 and Amazon 16, but less so with the other seven isolates (data not shown). A genomic segment of approximately 3000 nucleotides of this cassava virus were identified using the cDNA clone CFSV S5 as a hybridization probe (Figure 1.5.2B). The dsRNA for these hybridization experiments were run in agarose gels using DNA markers. The estimated size of the S5 segment was 3000 nucleotides as compared with the estimate of 2700 nucleotides when the size estimate was made in polyacrylamide gels using dsRNA markers.

Using primers designed from the cDNA S4 clones, the PCR products were amplified from either leaves or root tissues in nine isolates of CFSD but were not amplified in healthy controls (Figure 1.5.2C). The PCR products were transferred to membranes and visualized using the largest CFSV-S4 clone as the hybridization probe. It detected specific products of the expected size of 1000 bp from the leaf tissues in the CFSD affected plant but did not detect any products in the healthy controls (Figure 1.5.2D). This experiment was repeated using extracts from root tissues and the genomic S4 segment and the rt-PCR products were detected in all of the isolates tested.

Table 1.5.1. A comparison of the known size of the genomic segments of rice ragged stunt virus (RRSV) with the estimated size of the cassava frogskin virus genomic segments.

Genomic Segment No.	RRSV Size (bp)	CFSV Size (M_r) expressed as bp
1	3849	4000
2	3810	
3	3669	3700
4	3823	3900
5	2682	2600
6	2157	1800
7	1938	1750
8	1814	1700
9	1132	1100
10	1162	1000

The 70 nm virus-like particles and inclusion bodies found in CFSD affected cassava plants are similar to the virions and the inclusion bodies that are characteristic of plant viruses in the family *Reoviridae* (Nuss & Dall, 1990, Advances in Virus Research 38: 249-306). While most of the plant reoviruses are restricted

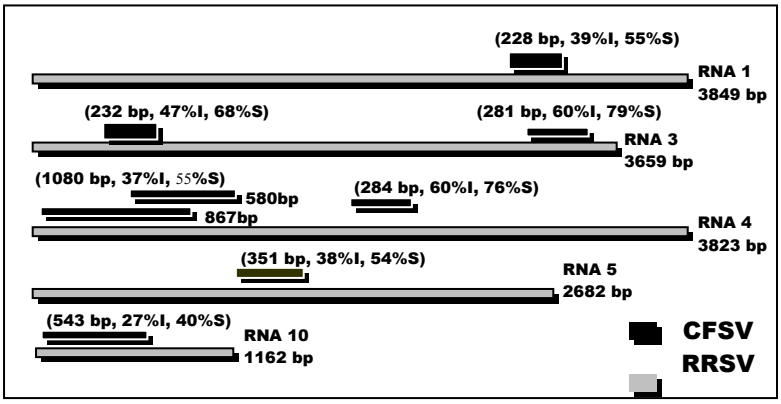


Figure 1.5.1. A comparison of the putative amino acid sequences deduced from cDNA clones of a virus found in cassava. The putative protein of CFSV-S4 is compared with the protein encoded by the segment 4 of rice ragged stunt virus.

to phloem or neoplastically developed phloem-derived tissues (Frison & Feliu, 1991, Technical guidelines for the safe movement of cassava germplasm. FAO/IBPGR), rice dwarf phytoreovirus (RDV) is found in both phloem and parenchyma cells. In most cassava landraces, the virus-like particles associated with CFSD were found only in phloem or associated cells of the affected plants. In the cassava landraces that developed leaf symptoms, virus-like particles and inclusion bodies could be found in many cell types, including the parenchyma cells of the leaves. CFSD is characterized by the hyperplasia of the root cortex. The fissures in the cortex of the cassava roots are similar to the symptom of longitudinal splitting in roots of maize infected with maize rough dwarf fijivirus (Milne & Lovisolo, 1977, Advances in Virus Research 21: 267-341).

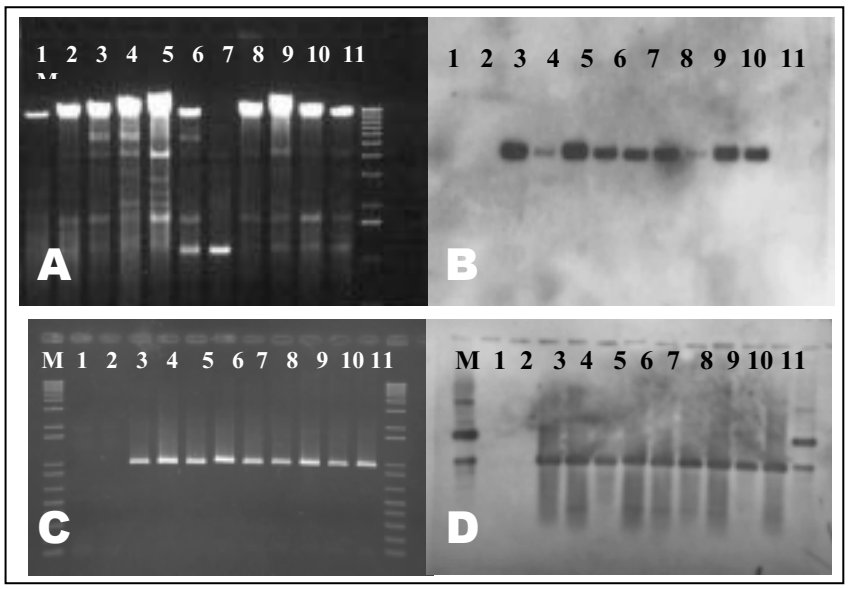


Figure 1.5.2. A series of dsRNA extractions and rt-PRC amplifications with their hybridizations using a cDNA probe derived from the segment S5: A) dsRNA Profiles isolated from healthy and affeted varieties in 1% agarose gel stained with ethidium bromide, B) hybridization using a cDNA probe derived from the segment S4, C) rt-PCR products using CFSV-S4 primers from healthy and CFSD affected varieties in 1% agarose gel stained with ethidium bromide, D) hybridization with the S4 probe. Healthy varieties: 1: Secundina, 2: CMC40. CFSD affected varieties: 3: Secundina 5, 4: Secundina 80, 5: Valluna 29, 6: CM-5460-10, 7: SM 909-25, 8: Regional Tolima, 9: CMC40, 10: Amazonas 16, 11: Catumare Jamundi, M: 1Kb + DNA molecular marker.

The virus-like particles found in the partially purified preparations were 45-50 nm in diameter and are similar to the core particles found during purification of other reoviruses (Pineda *et al*., 1983, ASIAVA 4: 10-12; Upadhyaya *et al*.,1998, Arch. Virol. 143:1815-1822). These particles were labile and were disrupted if subjected to additional steps of centrifugation. The size and fragility of these virus-like particles are similar to the plant reoviruses sub-viral particle that consists of double stranded nucleic acids and the inner shell or core (Bozarth & Harley, 1976, Biochim. Biophys. Acta 432: 329-335).

The relative molecular weights (M_r) of the dsRNAs found in CFSD affected plants are similar to those of RRSV (Collinge & Boller, 2001, Plant Mol. Biol. 46: 521-529), but only nine species of ads-RNA could be visualized using polyacrylamide gel electrophoresis. It was first reported that there were only eight genomic segments of RRSV, but the molecular characterization confirmed that genome of the RRSV consists of 10 species of dsRNA. It is expected that the existence of the 10 species of this cassava virus will be resolved through the molecular analysis of the genomic segments.

The use of RAPD and AFLP analyses to detect genetic differences is well documented and rt-AFLP is used as a means to identify differentially expressed genes (Kimura *et al*., 2001, Biosci. Biotechnol. Biochem. 65: 125-1283). Cytokinin-induced gene expression in cultured green cells of *Nicotiana tobacum* identified by fluorescent differential display). The successful cloning of a portion of this plant virus genome demonstrates that rt-RAPD and rt-AFLP can be useful techniques to characterize elusive causal agents.

Based on the amino acid similarity of putative proteins of portions of the segment 1, 3, 4, 5 and 10, the virus affecting cassava is most similar to the RRSV in the genus *Oryzavirus* of the family *Reoviridae* (Lozano & Nolt, 1989, Plant Protection and Quarantine: Selected Pests and Pathogens of Quarantine Significance 2:174-175; Upadhyaya *et al*., 1997, Arch Virol. 142:1719-26; Vos *et al*.,1995, Nucleic Acids Res. 23: 4407-4414). The two members of the Genus *Oryzavirus* are RRSV and ERSV, but there is sequence information only for RRSV, and it has little similarity with the reoviruses in the other genera. Given the geographical separation of the range of RRSV and its distant relation with other reoviruses, it is surprising that this cassava virus is related to it. The nucleotide or amino acid sequence analysis, of the eight cDNA clones of this cassava virus, revealed only significant similarity for RRSV.

The genomic segments of this cassava virus were identified in hybridization studies using cDNA clones. Also, the cDNA clones can detect these genomic segments in all nine isolates of CFSD tested to date, but they cannot be detected in CFSD free cassava. There are consistent differences in the intensity of the hybridization with the different isolates and this suggests that they may be significant variation between the isolates of this virus. Nevertheless, the virus can only be detected in CFSD affected plants, which suggests that this virus is associated with cassava frogskin disease.

Activity 1.6. Transmission of cassava frog skin disease: evaluation of homopteran species as vectors.

Contributors*:* Ma.P. Hernandez and A.C. Bellotti

Highlight:

∉ Several Delphacidae species, possible vectors of CFSD, collected from cassava fields infested with CFSD.

Rationale

Cassava frog skin disease (CFSD) is native to the Neotropics, probably originating the Amazon region of South America. It causes reductions in cassava root yields and hinders the movement of germplasm within and between countries. Detection of the disease is made difficult due to the lack of foliar symptoms on most cassava varieties. The disease has been described as a virus of the Family Reoviridae and/or a phytoplasm. The epidemiology of CFSD is still being studied. CFSD dissemination by infected planting material (stem cuttings) is well documented but the roll of an insect vector has not been adequately determined. Although previous research indicated the involvement of a whitefly (*Bemisia tuberculata*) vector, this is being questioned and re-evaluated (Calvert and Thresh, 2001, *In*: Hillocks *et al*., (eds), Cassava: Biology, Production and Utilization, CABI Publishing, pp 237 - 260). The beginnings in 2002 field surveys have been conducted in several regions of Colombia to collect possible vectors of CFSD. Emphasis is being given to homopteran species, especially those of the family Delphacidae, which are known vectors of Reoviruses. The objective of these studies is to determine the actual vector of CFSD (See CIAT PE-1 Annual Reports, 2003, 2004).

Materials and Methods

During the last trimester of 2004 and the early months of 2005, homopteran specimens from the families Delphacidae and Cixiidae were collected from cassava fields known to contain CFS diseased plants. Specimens were collected from six localities in the Valle del Cauca, Cauca, Quindio and Risaralda departments of Colombia. Additional samples were collected from vegetation in fields adjacent to cassava fields.

Collected insect specimens were brought into the laboratory and prepared for taxonomic identification. Preliminary identification can be done at CIAT while specimens are also sent to Dr. Stephen Wilson of the University of Missouri, a specialist in the families, for further verification.

Results and Discussion

Delphacidae species collected at CIAT, Palmira, were identified in the CIAT laboratory as *Peregrinus maidis* (Ashmead), *Sogatella kolophon* kirkaldy and *P. saccharicida* (identifications were corroborated by Dr. Stephen Wilson) [Table 1.6.1]

P. saccharicida was also collected from fields in the Rozo municipality. Adults of *P. maidis* were collected from several experimental fields at CIAT where CFSD was present. It should be noted that several cassava fields were weedy, especially with *Rottboellia exalta* (caminadora) and all nymphal instars of *P. maidis* were captured from this species. When weeds were removed, additional collections were made from these fields and high populations of *P. maidis* were again collected. This indicates that *P. maidis* can successfully feed on cassava in the absence of its preferred host.

The presence of *P. saccharicida* in cassava can be explained by the considerable cultivation of sugarcane on the CIAT farm. The ability of these species to successfully feed and reproduce on cassava needs to be determined.

Table 1.6.1. Homopteran species associated with the cassava.

Dept.	Municipality	Locality	Host	Family	Crop species
Valle del Cauca	Palmira	CIAT	*M. esculenta* *Rotboellia exaltata*	Delphacidae	*Peregrinus maidis* (Ashmead)
			M. esculenta *Digitaria sp*	Delphacidae	*Sogatella kolophon* Kirkaldy
			M. esculenta		*Perkinsiella saccharicida*
			M. esculenta	Cixiidae	*Oliarus* sp.
	Rozo		*M. esculenta*		*P. saccharicida* *Oliarus* sp.
Cauca	Santander de Quilichao	Granja CIAT	*M. esculenta* *Digitaria sp*		*S. kolophon*
			M. esculenta		*Oliarus* sp.
Quindío	Armenia	Marmato	*M. esculenta*		*S. kolophon* *S. molina*
Risaralda	Morelia	Sta . Rita	*M. esculenta*		*Oliarus* sp
	Cerritos		*M. esculenta*		*Oliarus* sp.

Identifications verified by:Dr. Stephen Wilson (Central Missouri State University).

S. kolophon was collected from several of the sites surveyed, while *S. molina* was only collected from fields in Armenia, Quindio. *Cixiidae* species are considered as possible vectors of phytoplasmas, but few species have been studied. *Oliarus* sp was collected from most of the sites surveyed; it would be

interesting to evaluate its relationship to cassava and CFSD since nymphs are found feeding on cassava roots.

Activity 1.7. Rearing of Delphacidae species, possible CFSD vectors on natural hosts.

Contributors: Ma.P Hernández and A. C. Bellotti

Highlight:

- Two Delphacidae species, *Peregrinus maidis* and *Sogatella kolophon,* possible vectors of CFSD successfully reared on gramineous hosts in the greenhouse.

Rationale

The search of an insect vector of CFSD has, in recent years led to the identification of numerous homopteran species found in association with the cassava crop. With the exception of whiteflies, mealybugs and scale insects, none of these are considered economic pests of cassava, causing yield losses. Most species collected belong to the homopteran families of Cicadellidae, Delphacidae and Cixiidae (See CIAT PE1 Annual Reports 2002, 2003 and 2004). However, species within these three families are known vectors of virus or phytoplasm diseases. Cassava is often grown in association with other crops and cassava fields will often contain several weed species that could be the preferred hosts of these homopterans. It is therefore necessary to determine if these insect species can successfully infest cassava and hence be a possible vector of CFSD. The objective of this research is to develop rearing methods for two Delphacidae species collected from cassava, first on their native hosts and secondly evaluate their ability to colonize cassava.

Materials and Methods

Nymphs and adults of *P. maidis* and *S. kolophon* (Fam. Delphacidae) were collected from field plantings of cassava at CIAT, Palmira and Santander de Quilichao, Cauca, during the first trimester of 2005. Specimens were also collected from weeds adjacent to the cassava plots. Field captures were done with the aid of a sweep-net or a buccal aspirator. Specimens were than placed in plastic boxes containing cassava leaves or grasses for live transportation to the laboratory.

Rearing is initiated in nylon meshed cages in the greenhouse. *P. maidis* is placed on six plants of its natural host, *Rottboellia exalta* and *S. kolophon* on its host *Digitaria sp*. (Figures 1.7.1 a, b). At the same time *P. maidis* nymphs and adults were placed in a nylon meshed cage containing both cassava and *R. exalta* (50% - 50%) in order to facilitate adaptation of *P. maidis* to cassava. Greenhouse conditions were; temp. 21 to 29∀C; 63-100% RH and 12:12 hrs photoperiod. Lifecycles studies for both species on their grass hosts were carried out in the greenhouse.

Results and Discussion

P. maidis and *S. kolophon* have been successfully reared on their natural hosts (*R. exalta* and *Digitaria sp,* respectively) in the greenhouse. Experiments to adapt these homopteran species to cassava have been discouraging. Both species prefer to feed on their natural gramineous hosts rather than on cassava. Maximum survival on cassava is only four days once the grass hosts are removed. Although *P. maidis* is oligophagous, the adaptation to a "new" host appears to be difficult. However, these studies will continue.

Biological characteristics of P. maidis: Hosts: this species is frequently found associated with maize, sorghum, millet, gramineous weeds and some shrubs and horticultural plants (Denno and Roderick, 1990, Annual Review Entomol. 35: 489-520).

Figures 1.7.1 a, b. *P. maidis* colonizing its native host *Rottboellia exalta* in the greenhouse.

Natural Enemies: Strepsitera parasites, possibly *Elenchus sp* were reared from field collected *P. maidis* individuals.

Geographic distribution: Widely distributed, especially in the tropical and sub-tropical regions.

Lifecycles studies: Under greenhouse conditions the egg to adult stages were 29-30 days. The egg stage is 10 to 12 days and nymphal development is 15 to 18 days. Females oviposit 17 $\pm$ 2.0 eggs per day and sex relation is 1.6 ♀ ; 1 ♂ .

Eggs are translucent, becoming more whitish as they develop. They are elongated, slightly curved near the cephalic region (Figure 1.7.2). Eggs are inserted in the central leaf vein.

1.7.2 **1.7.3** **1.7.4**

Figures 1.7.2-4. Eggs, nymph (N4) and adult *Peregrinus maidis* feeding on *Rottboellia exalta* leaves.

There are five nymphal instars; coloration during the first 3 instars is yellowish and dark spots appear on the thorax and abdomen during the latter instars (Figure 1.7.3). During the N4 nymphal stage the genus is distinguishable. It is common to find branchipterous individuals within the population, owing to conditions of confinement.

Adults can be characterized by their general coffee-yellow coloration with darkend areas around the distal zones of the first two antennal segments, on the terminal parts of the costal nerves and more diffuse on the dorsal-apical area and on the tergos, pleuras and exterior of the abdomen (Figure 1.7.4). Legs are yellowish with the coxas and femurs a dark brown.

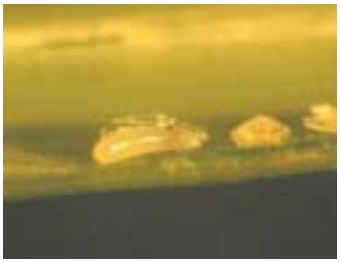
P. maidis is the recognized vector of five virus diseases, maize stripe, Iranian maize mosaic, maize mosaic, maize sterile stunt and finger millet mosaic, that affect maize and sorghum (S. Wilson, personell communication).

Biological characteristics of Sogatella kolophon: Hosts: Found on grasses, often at low densities. Sporadically recovered from maize and oats.

Natural Enemies: Similar to *P. maidis*, Strepsiptera parasites, possibly *Elenchus sp* recovered from field collected specimens.

Geographic distribution: Amply distributed, including the eastern Pacific, the Ethiopian region and in the new world it is reported from the USA, Mexico, Bermuda, Guyana and Argentina. This is the first established report from Colombia.

Lifecycle studies: The egg to adult stage under greenhouse conditions was 26 to 39 days. Egg eclosion is in 10 days and the average nymphal period is 15 to 17 days. Eggs are inserted in the central leaf vein and stem by using the ovipositor as a sword. Eggs are cream colored, turning more yellowish as they advance in development. Although eggs are similar to *P. maidis,* they are smaller (Figure 1.7.5).



1.7.5

1.7.6

1.7.7

Figure 1.7.5-7. Eggs, nymph (N4) and adult of *Sogatella kolophon* feeding on *Digitaria* sp. in the greenhouse.

Nymphs have five instars, are clear yellow in color, becoming reddish as development advances (Figure 1.7.6). Adults of this species are easily distinguishable by the presence of a whitened longitudinal dorsal band on the vertex and thorax of the pronotum. The tegman is translucid with a dark spot on the apical-dorsal area.

Sexual dimorphism occurs in color and size; females are a clear yellow in color (Figure 1.7.7) while males are dark brown. *S. kolophon* is considered to be the vector of Brasilian wheat spike disease, Digitaria striate virus and maize sterile stunt virus.

Activity 1.8. Evaluation of *Peregrinus maidis* and *Sogatella kolophon* as possible vectors of CFSD.

Contributors: Ma.P Hernández and A. C. Bellotti

Highlights:

- Planthoppers species are the primary reovirus vectors.
- A two field collected species suspected of being reovirus vectors in cassava crops: *S. kolophon* and *P. maidis.*
- Studies of transmission were carried out with healthy insect raised in the laboratory; different acquisition, latency and inoculation periods were test.
- Virus detection in inoculated plants have so far been unsuccessful

Rationale

The causal agent of cassava frog skin disease has been described as a virus from the family Reoviridae. Reoviruses are known to be transmitted or vectored by homopterans of the family Delphacidae. There is a hypothesis that reoviruses of plants are originally from insects (Noda and Nakashima, 1995, Seminars in Virology 6: 109-116). According to results reported by the CIAT Virology group (PE1 Annual Report 2004) the virus found in CFSD infected materials shows a 72% homology with Rice ragged oryzaviruses (RRSV). The Delphacidae is an economically importantly family within the Superfamily Fulgoridae; most species feed on monocodyledonous plants and are known vectors of viruses in maize, sugarcane, rice and other crops (Denno and Roderick, 1990, Annual Review Entomol. 35: 489-520). There are nearly 50 genera and about 255 known species in South America, compared with 290 species reported from Mexico.

The objective of this research is to evaluate these Delphacidae species as possible vectors of CFSD in healthy cassava, var. Secundina.

Materials and Methods

Virus acquisitions were conducted by first selecting 50 N3 and N4 nymphs from established colonies of P. *maidis* and *S. kolophon* in the greenhouse. These were allowed to feed on two-week cassava plants that showed marked symptoms of CFSD. These plants originated from stem cuttings of plants naturally infected with CFSD in the field that displayed typical root symptoms. This should guarantee the recovery of native strains of the CFSD virus.

The virology unit verified the presence of the CFSD pathogen in the plant, when the stem cuttings were selected. Samples were taken from the youngest leaves of the selected plants. The insects feed on cassava plants for 48 hours to acquire the virus. After this period the Virology Unit examined five individuals using PCR.

Individuals feeding on CFSD infected plants were separated into two groups: half were placed on *Digitaria sp.* plants (*S. kolophon*) and *Rottbollia (P. maidis)* for an initial period of 5 days. The second group were placed on healthy Secundina plants for 48 hours or until insect death. It is not known if these species can transmit the virus immediately after feeding on infested plants. To insure feeding the methodology used employed small cages that confined the insect to a specific site on the cassava plant. Nine cassava plants were used in the transmission trial and nine plants were employed as controls and held under the same conditions.

Inoculated plants were placed in the Virology Unit greenhouse under controlled conditions to await expression of CFSD symptoms. Plants were observed daily during a one month period or until the appearance of symptoms (Figure 1.8.1).

Results and Discussion

At present CFSD virus transmission has not been achieved as Secundina plants do not show leaf symptoms. During the course of these evaluations, changes in methodologies have occurred, but it is recognized that longer observational periods may be required as well as more detailed studies with these organisms.

Molecular studies with those individuals that have fed on plants with CFSD symptoms have not provided conclusive results. It is considered that the PCR methodology that was designed for plants will need to be further adjusted for virus detection in these insects.

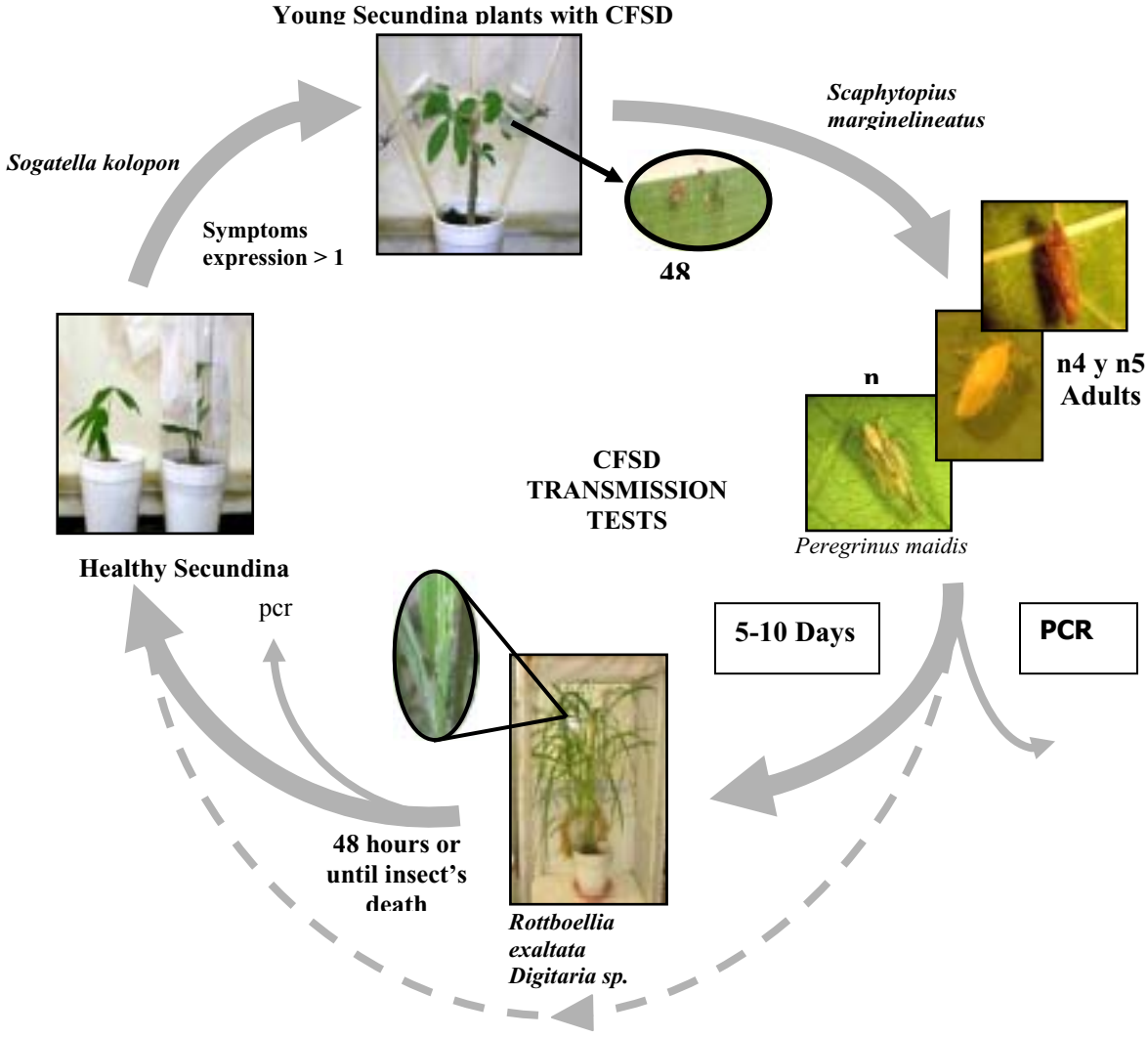


Figure 1.8.1 Cassava Frog Skin disease transmission test scheme

Activity 1.9. Evaluating the influence of soil as a source of cassava frog skin disease vectors

Contributors: E. Álvarez, J. F. Mejía, and J. Loke

Rationale

Phytoplasmas arze a group of bacteria that lack cell walls. They are obligate pathogens of a wide range of different plants. Phytoplasmas can spread through infected plant material or insect vectors. Because phytoplasmas do not grow on artificial media, studying them is extremely difficult, and many questions about their biology, spread, pathology, and control remain unanswered.

Phytoplasmas are spread mainly by cicadas and planthoppers, which have sucking-piercing mouth parts (order: Hemiptera, families: Jassidae, Cixiidae, and Psyllidae). Both adults and nymphs can spread phytoplasmas. Host specificity is variable between vector species. Some vectors are polyphagous and feed on a variety of different host plants, for example, Hyalesthes obsoletus on grapevine (giving rise to "bois noir"). In general, three types of host plants can be distinguished: those on which vectors feed throughout their whole life cycle (both imagos and larvae); those on which imagos (but not larvae) feed regularly; and those on which imagos feed only arbitrarily.

The vectors cannot transmit the phytoplasmas immediately after feeding on an infected plant, but only after incubation, the length of which depends on temperature, among other parameters. During incubation, the phytoplasmas multiply and spread within the insect. No vertical infection of insects is known, meaning no phytoplasmas are transmitted from one generation to the next one via infected eggs. Phytoplasmas also have been found in some insect groups that most likely do not transmit them. In these insects, phytoplasmas either do not multiply or transmission to plants is inefficient because the mouth parts are different.

Phytoplasmas can survive in the soil; they are also found in certain soilborne insect stages and in living roots, but are unlikely to survive in pure (mineral) soils and dead plant material. Our study aimed to evaluate the soil as a possible source of microorganism vectors of cassava frogskin disease (CFSD), and whether the presence of aerial vectors is related to the dissemination of this important disease.

Materials and Methods

In Santander of Quilichao (Cauca, Colombia), a field trial was established with cassava variety HMC-1, using stakes from infected plants (Jamundí, Valle del Cauca, Colombia) and healthy plants (Montenegro, Quindío, Colombia). To cut the stakes, a machete was disinfected in a solution of 1% sodium hypochlorite. The plants were planted inside and outside a screen house.

The outside plants were separated into two blocks, one receiving a weekly application of insecticides to control aerial vectors and the other no applications. Plots of 12 plants were established in a randomized complete block design with 3 replications, where the main plots were with insecticides and without insecticides. The plants inside the screen house received weekly applications of insecticides, rotating two products: either Sistemin® (dimethoate, 3 cc/L of commercial product) or Malathion® (malathion, 1 cc/L of commercial product). The subplots, both inside and outside the screen house, were plants from healthy seed and plants from seed infected with CFSD.

On day 30 after planting, stake germination was evaluated and records periodically taken on the presence of pests and diseases. When the plants were 6 months old, the presence or absence of symptoms of CFSD was recorded for roots in a sample of 3 plants per plot. In addition, samples of roots and leaves were collected for detecting phytoplasmas through nested PCR, using the methodology described in Activity 2.

When the plants were 10 months old, cuttings from 3 plants in each plot were taken to root in water and observe the expression of symptoms in leaves under laboratory conditions (23–30 °C). At 11 months, all the plants were harvested, symptoms in roots recorded, and DNA extracted for detecting phytoplasmas through PCR.

Results and Discussion

Plants from infected stakes presented symptoms of CFSD, whereas plants from healthy seed did not show symptoms.

Variety HMC-1 expressed leaf symptoms (chlorosis and mild curling) (Figure 1.9.1) in the cuttings that rooted under laboratory conditions. The plants with symptoms in the laboratory were from plants that expressed CFSD in the field, at a correlation of 0.82.

Figure 1.9.1. Leaves from cassava plants grown from cuttings rooted in the laboratory. The cuttings were taken from both healthy and diseased plants growing in a trial established in the field with variety HMC-1. The leaves show different degrees of severity of cassava frogskin disease: (A) healthy plant; (B) initial symptoms presenting minor chlorosis and curling in some leaf lobes; and (C) severe symptoms of chlorosis and curling.

Table 1.9.1 illustrates the different degrees of severity of disease per plant observed during evaluations at harvest and the symptoms expressed by the laboratory cuttings. The evaluation by nested PCR for phytoplasma was carried out for leaves and roots obtained from plants established in the field. A correlation of 0.37 was obtained between the degree of severity and detection by PCR. This correlation is high if we take into account that many of the plants from healthy seed and evaluated as healthy at harvest may possibly have acquired the pathogen and only in the following cycle would the symptoms be observed, thus demonstrating the technique's potential to carry out early detection of the pathogen in plants that as yet do not express symptoms.

To correlate presence of the disease and detection by PCR for phytoplasmas, the frequencies of amplification were analyzed between the number of plants amplified by plot according to treatment (i.e., outside screen house and not fumigated; outside and fumigated; inside and fumigated weekly with insecticide—*see* Table 1.9.1).

Table 1.9.1. Evaluation of different treatments in the field in terms of severity of disease, symptoms in leaves, and nested PCR for cassava frogskin disease.

Plot	Treatment[a]	Rep.	Source of seed[b]	Germin -ation	Population (%)	Severity per plant[c]	Symptoms in cuttings/plant[d]	PCR[e] (+/-)
1	Outside, not fumigated	1	D	100	100	4, 4, 4	nd, Cl, nd	3/3
2	Outside, not fumigated	1	H	91.7	83.3	1, 1, 1	nd, nd, nd	1/3
3	Outside, not fumigated	2	D	100	100	3, 4, 3	Cl, nd, nd	2/3
4	Outside, not fumigated	2	H	100	83.3	1. 1, 1	Ns, nd, nd	1/3
5	Outside, not fumigated	3	H	100	100	1, 1, 1	nd, nd, Ns	3/3
6	Outside, not fumigated	3	D	100	100	4, 4, 4	nd, nd, nd	2/3
7	Inside, fumigated	1	H	91.7	91.7	1, 1, 1	Ns, Ns, Ns	0/3
8	Inside, fumigated	2	H	91.7	83.3	1, 1, 1	Ns, Ns, Ns	1/3
9	Inside, fumigated	3	H	100	91.7	1. 1, 1	nd, nd, nd	0/3
10	Inside, fumigated	1	D	58.3	41.7	2, 2, 4	Ns, nd, nd	1/3
11	Inside, fumigated	2	D	83.3	58.3	2, 2, 2	Cl, Mc, Cl	2/3
12	Inside, fumigated	3	D	58.3	50	4, 4, 2	Ns, nd, nd	1/3
13	Outside, fumigated	1	D	100	91.7	4, 2, 4	nd, Mc, nd	2/3
14	Outside, fumigated	1	H	100	91.7	1. 1, 1	Ns, Ns, Ns	1/3
15	Outside, fumigated	2	D	100	100	4, 4, 2	Cl, Cl, Mc	2/3
16	Outside, fumigated	2	H	100	91.7	1, 1, 1	Ns, Ns, Ns	1/3
17	Outside, fumigated	3	D	100	100	2, 3, 4	Ns, Cl, Cl	3/3
18	Outside, fumigated	3	H	100	100	1, 1, 1	Ns, Ns, Ns	0/3

a. Outside = cassava plants were planted in the field; Inside = cassava plants were planted inside a screen house.
b. Seed evaluated at harvest according to symptoms in root: D = diseased seed (Jamundí, Valle del Cauca); H = healthy seed (Montenegro, Quindío).
c. Three plants per plot evaluated for diseased roots at harvest (September 2005) according to a disease severity scale, where 1 = no symptoms; 2 = mild; 3 = moderate; 4 = moderate to high; 5 = severe symptoms.
d. Evaluation of symptoms in cuttings, where Ns = no symptoms; Cl = chlorosis; Mc = minor chlorosis; nd = not determined.
e. Primers used for the evaluation were P1/P7–FSD-F/R and R16F2/R2–R16(III)F2/R1. Two evaluations were made, one in May and another in September at harvest. Values refer to number of replications out of 3 where detection was successful.

The results of the study to detect phytoplasmas through PCR were as follows:

- *In the plots with plants from healthy seed* (plots 2, 4, and 5; outside and not fumigated), the frequency of positive samples was 55% (i.e., 5 positive plants out of a total of 9 in the 3 plots), 11% (plots 7, 8, and 9; inside), and 22% (plots 14, 16, and 18, outside and fumigated).

- *In the plots with plants from diseased seed*, the frequency of positive samples was 77% (plots 1, 3, and 6; outside and not fumigated), 44% (plots 10, 11, and 12; inside), and 77% (plots 13, 15, and 17; outside and fumigated) (Table 1.9.1).

The absence of phytoplasmas through amplification of DNA obtained from plants in the plots with healthy seed and the application of insecticides indicates that insect vectors of phytoplasmas may exist.

The number of phytoplasmas detected in plants in the plots inside the screen house, both in healthy and diseased seed, was low, compared with the plots outside the screen house. The few infected plants from healthy seed inside the screen house may have been infected through homopterous species of insects of the Cixiidae family. These planthoppers, in their subterranean nymphal stage, feed on grass roots, and have been reported as vectors of phytoplasmas. An example is *Myndus crudus* Van Duzee on coconut palms; nymphs develop at or just under the soil surface on grasses or sedges, while adults feed on palms. At least 37 species of grasses (Gramineae) and 4 species of sedges (Cyperaceae) have been reported as hosts to nymphs of *M. crudus* (Howard and Ropeza, 1998, Florida Entomol 81:92–97).

The rate of detection of phytoplasmas in the plots with plants infected by CFSD and healthy plants is high, considering that a rate of no detection of phytoplasmas is possible in plants presenting symptoms typically associated with them. Lack of detection could be attributed to substances in plant-tissue extracts inhibiting amplification, irregular distribution of phytoplasmas in the plant, low concentrations of the microorganism, or environmental effects, as occurred with cv. Manzano with apple proliferation phytoplasma, where proliferation is often disseminated in scion wood. Although the causal agent does not appear systemic, trees may yield a high proportion of apparently healthy but infected buds.

Distribution of phytoplasmas in the tree is not constant over the year. In winter, the content of phytoplasmas declines in the tree as a result of sieve tube degeneration. They also concentrate more in the roots but, during April to May, they reinvade the stem from the roots and reach a peak in late summer or early autumn. The distribution pattern of the phytoplasmas in the tree is also dependent on temperature. In France, phytoplasmas could be found throughout the trees at temperatures of 21–25 °C, causing symptoms. At 29–32 °C, symptoms were inhibited and phytoplasmas were found only in the roots, but reinvaded the stems when plantlets were stored at lower temperatures (EPPO/CABI 1996).

When a tree is inoculated with an infected bud, the first symptoms appear the following year, mostly on the inoculated branches. When carried in the rootstock, the causal agent produces symptoms on the first growth of the scion. It appears to be localized mainly in suckers and terminal shoots, where it can be found in the phloem of leaf petioles, midribs, and stipules (EPPO/CABI 1996).

From this same trial, we carried out a replication with cuttings to reduce the number of dead plants and be able to complete the data as yet not determined. We are also evaluating cuttings from 30 varieties with and without symptoms in the field under laboratory conditions to confirm the results obtained.

Activity 1.10. Studying the transmission of a phytoplasma belonging to the 16SrIII group (cassava frogskin disease phytoplasma; Cfdp) associated with cassava frogskin disease (CFSD)

Contributors: E. Álvarez, J. F. Mejía, and J. Loke

Highlight:

- Characteristic symptoms of foliar frogskin disease (FSD) were developed in the parasitic dodder plant (*Cuscuta* sp.) after infection by a pathogen transmitted from infected *in vitro* plants of cassava variety Secundina. This result provided evidence that FSD is caused by a phytoplasma.

Rationale

Definitive proof that a microorganism causes a disease is obtained by fulfilling Koch's postulates. However, because phytoplasmas are not cultivable, the fulfillment of Koch's postulates is very difficult. In general, phytoplasmas are believed to cause a certain plant disease if the plant shows typical "phytoplasma-related" symptoms, and phytoplasmas can be detected regularly in the diseased plants. Our study aimed to examine the transmission of a phytoplasma of the 16SrIII group (Cfdp), associated with cassava frogskin disease (CFSD), thereby fulfilling Koch's postulates. This may confirm that a phytoplasma is the causal agent of the disease.

Materials and Methods

The transmission of the pathogen was carried out on two cassava varieties infected with CFSD and evaluated with PCR as positive to Cfdp, using the ectoparasite *Cuscuta* sp. as a bridge transmitter and grafts (clefts and splices) into periwinkle (*Catharanthus roseus* (L.) G. Don) and healthy cassava.

The cassava variety used was SM 909-25, selected for severe symptoms in roots and the presence of symptoms—chlorosis and curling—in leaves under greenhouse conditions (23 °C and 80% RH) (Figure 1.10.1a). A clone (CW 94-21) from the CW family in CIAT's cassava genetic improvement program was also used. It had been found in the field with the characteristic symptoms of the disease in roots.

For transmission with *Cuscuta*, sexual seed was planted in soil, together with the cassava plants corresponding to each treatment. Once germinated, the seeds were connected with each other through the long extensions issued by the parasite (Figure 1.10.1b). Periwinkle seedlings were obtained from sexual seed and the plants ascertained as free from phytoplasmas through nested PCR . Transmission time was estimated from the establishment of *Cuscuta* in the plants corresponding to each treatment. The transmission period was about 2 months under greenhouse conditions (20–25 °C and 50%–90% RH). For transmission, the following treatments were carried out: (1) from infected cassava plants to healthy periwinkle plants; (2) infected periwinkle plants to healthy periwinkle plants; (3) infected cassava plants to healthy cassava plants; (4) healthy periwinkle plants to healthy periwinkle plants; and (5) healthy cassava plants to healthy periwinkle plants.

Transmissions by graft were carried out with cleft and splice grafts, using the leaf central nervure and terminal shoots of infected cassava plants. As stock, we used 2-month-old cassava plants and 5-to-6-week-old periwinkle plants. The treatments for this trial were (1) infected cassava plants to healthy periwinkle plants; (2) healthy periwinkle plants to healthy periwinkle plants; and (3) healthy cassava plants to healthy periwinkle plants. Each treatment was carried out with 6 replications. Once the *Cuscuta* plants were established and the grafts had developed well, monthly evaluations by PCR and by characteristic symptomatology reported in periwinkle were carried out.

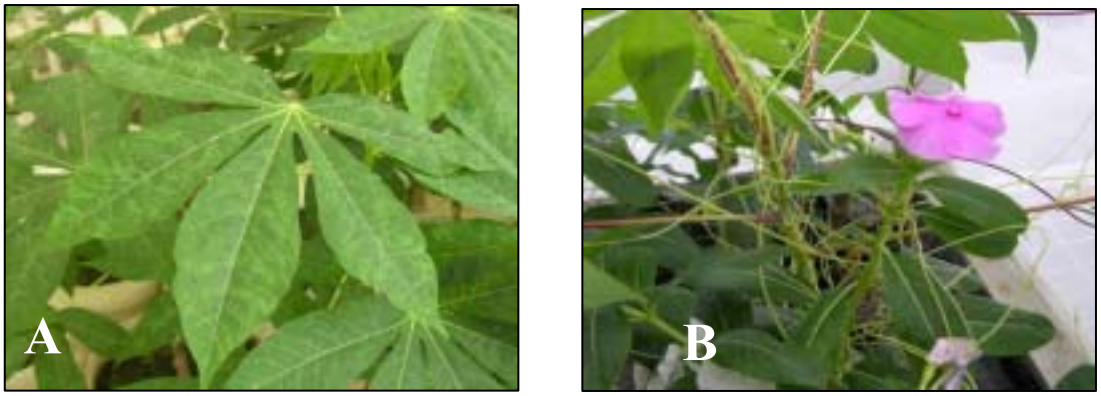


Figure 1.10.1. (A) Cassava variety SM 909-25, which is highly susceptible to cassava frogskin disease, showing leaf symptoms of chlorosis and curling under greenhouse conditions. (B) *Cuscuta sp*. parasitizing cassava and periwinkle, and acting as a bridge transmitter of phytoplasmas.

Results and Discussion

After 4 months of exposure, the pathogen's transmission was achieved through *Cuscuta* from infected cassava plants to healthy periwinkle plants and from infected to healthy periwinkle plants (treatments 1 and 2). Detection was achieved with nested PCR in 2 of the 6 replications for each treatment with variety SM 909-25. For the other three treatments and the other variety, positive amplifications were not obtained (Table 1.10.1).

Transmission by graft was generally high for treatment 1—of the 6 replications, 66% were detected as positive by nested PCR in the two infected varieties (SM 909-25 and CW 94-21). For the other two treatments, no positive samples were detected. Phytoplasmas were detected, using grafts, 3 to 4 months after exposure (Table 1.10.1).

So far, we have not observed the characteristic symptoms caused by phytoplasmas in periwinkle, even though we detected the presence of phytoplasmas with the nested-PCR technique in infected cassava plants and later in periwinkle plants infected through *Cuscuta* or grafting. A major cause is the rather unfavorable environmental and greenhouse conditions for symptom expression.

Valencia *et al*. (1993, Fitopatol Colomb 17:39–45) in studies on the transmission of the causal agent of witches' broom in cassava, caused by a phytoplasma, achieved 100% transmission between cassava and periwinkle, using *Cuscuta* sp. and grafts, after 3 months of exposure. This demonstrated that transmission of phytoplasmas between these two different species is possible. These studies reported the expression of symptoms under growth chamber conditions with temperatures at 18–20 °C and RH at 44%–84%. The symptoms were not very severe, but vegetative depressions were observed in less than 6 months since transmission began.

The periods of incubation and optimal greenhouse conditions are fundamental for the expression of symptoms characteristic of phytoplasmas in periwinkle plants. The plants evaluated as positive by PCR will be exposed to different periods and greenhouse conditions to seek the optimal for reproducing symptoms.

Table 1.10.1. Results obtained with *Cuscuta* sp. and grafts as transmitters of phytoplasmas from cassava infected with Cfdp to periwinkle.

Treatment		PCR (+)[a]	
		SM	CW
No.	Description	909-25	94-21
Cuscuta sp.			
1	Infected cassava to healthy periwinkle	2/6	0/6
2	Infected periwinkle to healthy periwinkle	2/6	0/6
3	Infected cassava to healthy cassava	0/6	0/6
4	Healthy periwinkle to healthy periwinkle	0/6	0/6
5	Healthy cassava to healthy periwinkle	0/6	0/6
Grafts (clefts and splices)			
1	Infected cassava to healthy periwinkle	5/6	3/6
2	Healthy periwinkle to healthy periwinkle	0/6	0/6
3	Healthy cassava to healthy periwinkle	0/6	0/6

a. Values refer to number of replications out of 6, where detection was successful.

Activity 1.11. Evaluating specific primers for high-specificity detection of phytoplasmas associated with cassava frogskin disease (CFSD)

Contributors: E. Álvarez, J. F. Mejía, G. Llano, and J. Loke

Highlight:

∉ FSD-specific primers proved highly specific and sensitive for detecting the phytoplasma. The PCR assay designed provided an effective alternative to conventional tests.

Rationale

Recent research on phytoplasma diseases has focused on developing techniques for detecting the pathogens and diagnosing their associated diseases. Such techniques include assays with antibodies, genomic DNA probes, and, more recently, PCR-based DNA markers. Other focal areas of phytoplasma research have been genetic variation among phytoplasmas and phylogenetic relationships among phytoplasma groups and other Mollicutes. Using heterogeneous DNA probes and differential screening, the 16S ribosomal RNA (rRNA) gene and ribosomal protein genes have been cloned from a phytoplasma. Universal primers were then designed and used to amplify 16S rRNA gene segments from each known phytoplasma group. Overall classification of phytoplasmas and general phylogenetic relationships were derived from extensive studies of those conserved genes. The objectives of the present study are to achieve high specificity in detection, using nested PCR, in order to improve the detection of phytoplasmas in cassava plants presenting symptoms of CFSD, weeds, and potential insect vectors. A protocol will be standardized for detecting phytoplasmas of the 16SrIII group associated with CFSD in cassava by using specific primers.

Materials and Methods

We previously described obtaining complete sequences of DNA fragments through PCR from samples of two cassava varieties. They were reported to GenBank, which gave them accession numbers AY737646

and AY737647. We conducted analyses of homology with these sequences against 24 sequences of the 16SrIII group and accessions of phytoplasmas representing at least 14 primary phytoplasma groups, using multiple alignments among the sequences.

Specific differences in nucleotides were sought, seeking a series of bases that would be specific to the cassava phytoplasma. The homology of the sequences was calculated (in %) by taking the identical number of bases over the difference of aligned sequences and total size of gaps (in %). "Gap (%)" is the number of gaps of all sequences over the size of aligned sequences.

The results of the phylogenetic and homology analyses showed that the CFSD phytoplasma clustered closely with other known X-disease (16SrIII) group strains, thus supporting its assignment to this group. We found multiple differences among the sequences of the CFSD phytoplasma and the 16SrIII-group phytoplasmas in the 16S rRNA gene, generating sufficient information to design primers (CIAT 2004). The primers designed were FSD-F (5'-TTT GAA GGT ATG CTT AAG GAG-3') and FSD-R (5'-GGA GTC CCG TCA ATT CCT T-3').

Standardizing primers: The principal objective for which the primers were designed was to avoid the need for nested PCR, using the primers directly and seeking to make detection more efficient, thus saving time and increasing the number of samples processed per day. Primers FSD-F/R were carried out in a total volume of 25 µL, containing 1X PCR buffer (supplied by the manufacturer of the polymerase), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 U of *Taq* polymerase (Promega, Madison, WI), and 0.5 pmoles of each primer. Reactions were heated at 94 °C for 2 min and then cycled through 35 cycles at 94 °C for 1 min, passing through 48, 50, 52, and 55 °C for 2 min each, and 72 °C for 3 min, followed by a final extension of 10 min at 72 °C. As positive controls, DNA of different cassava varieties infected with CFSD was evaluated as positive for phytoplasma with the universal primers and sequencing (Table 1.11.1) [CIAT, PE-1 Annual Report 2004].

Table 1.11.1. DNA evaluated with direct PCR, using specific primers FSD-F/R.

Sample code number	Variety	Tissue	PCR universal primers[a]
1	M Col 2063	Leaf	+
2	CM 6740-7	Leaf	+
3	SM 909-25	Leaf	+
4	SM 909-25	Root	+
5	SM 1219-9	Leaf	+
6	SM 1219-9	Root	+
7	M Col 2063 (Invitro)-1	Leaf petiole (healthy)	-
8	M Col 2063 (Invitro)-1	Stem (healthy)	-
9	M Col 2063 (Invitro)-2	Leaf petiole (healthy)	-
10	M Col 2063 (Invitro)-2	Stem (healthy)	-
11	Control (Sqmp)[b]	Leaf petiole	+
12	Control (Ccp)[c]	Petiole/stem	+

a. Samples positive to phytoplasmas through PCR with universal primers and sequencing.
b. Sqmp = *Solanum quitoense* machorreo phytoplasma (AY731819).
c. Ccp = coffee crispiness phytoplasma (AY525125).

DNA sequencing: The product amplified from direct and nested PCR was purified, following the protocol described for the QIAquick PCR Purification Kit, and sequenced. The fragments were sequenced, using the BigDye® Terminator Kit (Applied Biosystems) in an ABI PRISM® 377 DNA Sequencer. Sequence analysis was done with the programs Sequencher 4.1 and DNAMAN version 4.13. Homology was sought in the NCBI's GenBank (www.ncbi.nlm.nih.gov), using the tool BLAST®n.

Results and Discussion

Before evaluating the primers, their sequences were aligned with phytoplasma accessions of the 16SrIII group in GenBank (including those of cassava, AY737646 and AY737647) (Figure 1.11.1) to confirm that the primers' sequences were correct. On evaluating the different annealing temperatures (48, 50, 52, and 55 °C), two fragments of about 0.6 and 0.7 kbp were obtained for all DNA evaluated, except the positive controls Sqmp and Ccp of the 16SrIII group (Figure 1.11.2; Table 1.11.1), showing in-specificity of the primers.

Origin 1
ccctaagac gaggataacg attggaaaca gttgctaaga ctggatagga aaagtaaagg
61 catctttact tttttaaaag accttt tttg aaggtatgct taaggag ggg cttgcgacac
121 attagttagt tggcagggta aaggcctacc aagactatga tgtgtagctg gactgagagg
181 ttgaacagcc acattgggac tgagacacgg cccaaactcc tacgggaggc agcagtaggg
241 aatttt cggc aatggaggaa actctgaccg agcaacgccg cgtgaacgat gaagtacctc
301 ggtatgtaaa gttcttttat taaggaagaa aa aagagtgg aaaaactccc ttgacggtac
361 ttaatgaata agccccggct aattatgtgc cagcagccgc ggtaatacat aaggggcgag
421 cgttatccgg aattattggg cgtaaagggt gcgtaggcgg tttaataagt ctatagttta
481 atttcagtgc ttaacgctgt tgtgctatag aaactgtttt actagagtga gttagaggca
541 agcggaattc catgtgtagc ggtaaaatgc gtaaatatat ggaggaacac cagaggcgta
601 ggcggcttgc tgggacttta ctgacgctga ggcacgaaag cgtggggagc aaacaggatt
661 agataccctg gtagtccaca ccgtaaacga tgagtactaa gtgtcgggta aaaccggtac
721 tgaagttaac acattaagta ctccgcctga gtagtacgta cgcaagtatg aaactta aag
781 gaattgacgg gactcc gcac aagcggtgga tcatgttgtt taattcgaag atacacgaaa
841 aaccttacca ggtcttgaca ttttcttgcg aagttataga aatataatgg aggttatcag
901 gaaaacaggt ggtgcatggt tgtcgtcagc tcgtgtcgtg agatgttagg ttaagtccta
961 aaacgagcgc aacccttgtc gttaattgcc agcatgtaat gatggggact ttaacgagac
1021 tgccaatgaa aaattggagg aaggtgggga ttacgtcaaa tcatcatgcc ccttatgatc
1081 tgggctacaa acgtgataca atggttgata caaagagtag ctgaaacgcg agttcttagc
1141 caatctcaca aaa tcaatct cagttcgga t tgaagtctgc aactcgactt catgaagttg
1201 gaatcgctag taatcgcgaa tcagcatgtc gcggtgaata cgttctcggg gtttgtacac
1261 accgcccgtc aaaccacgaa agttggcaat accccaaa

Figure 1.11.1. Cassava frogskin disease phytoplasma strain FSDY29 16S ribosomal RNA gene, partial sequence, accession number AY737647. Yellow = primers designed for cassava frogskin disease phytoplasm (Cfdp); green = primers designed for 16S rRNA gene (X-disease phytoplasma group).

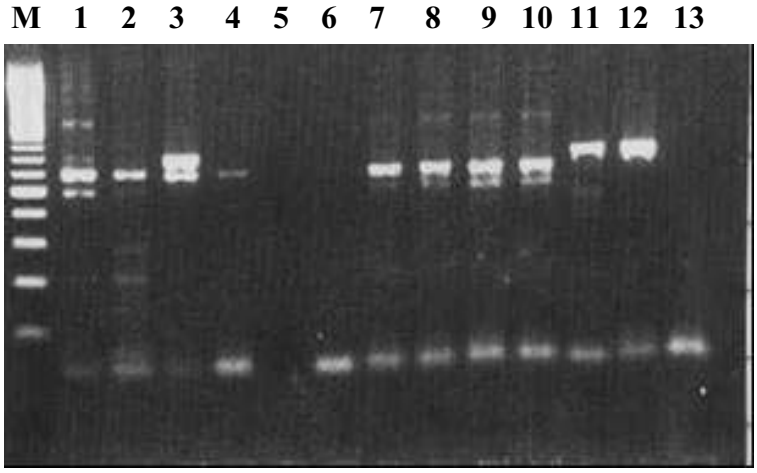


Figure 1.11.2. Two fragments, about 0.6 and 0.7 kbp long, were obtained through direct PCR with primers FSD-F/R. Lanes 1–10 = samples 1–10 (Table 1.11.1); lanes 11 and 12, respectively = positive controls Sqmp (*Solanum quitoense* machorreo phytoplasma) and Ccp (coffee crispiness phytoplasma); lane 13 = negative control; M = molecular weight marker of 1 kbp.

To confirm this result, the number of cassava samples was broadened, taking 32 samples of DNA from healthy and diseased plants of the varieties Manzana and ICA Catumare (8 healthy and 8 diseased of each variety), obtaining bands of the two sizes (0.6 and 0.7 kbp) in both healthy and diseased tissues, confirming what was evaluated with the samples described in Table 1.11.1 and Figure 1.11.2, very possibly because of in-specific amplifications with the plant's DNA (Figure 1.11.3). The 0.6 and 0.7-kbp bands were sequenced to confirm that the fragments that amplified to a molecular weight equal to that of the controls belonged to a phytoplasma and not to the plant's nuclear or chloroplastic DNA. For the 0.6-kbp fragments, we found homologies with 80% of accessions of *Lycopersicon esculentum* Mill. (CT476825) and with chromosome 5 of *Solanum demissum* Lindley (AC149301). In contrast, the 0.7-kbp fragments showed a 99% homology with cassava frogskin disease phytoplasma strain FSDY29 (AY737647).

Harrison *et al*, (2002, Plant Dis 86: 676–681) had similar problems of amplification, improved with the use of universal primers (P1/P7) that amplified genes 16S rRNA, 23S rRNA, and the 16S/23S spacer region from genomic DNA. These primers were used in the first cycle of a nested PCR, to be later nested with more specific primers or with primers designed for the phytoplasma identified. PCR amplification of the 16S rRNA gene and the 16S/23S spacer region from genomic DNA yielded one fragment of about 0.78 kbp, from cassava frogskin disease phytoplasm (Cfdp) isolates (Figure 1.11.4). Amplification fragments of the same size were obtained from the Sqmp and Ccp phytoplasmas. From 10 cassava samples, 6 showed positive amplification for the 16S rRNA gene. These gene segments were not amplified during PCR from healthy control plants. Similar results were obtained when the 32 DNA samples from varieties ICA Catumare and Manzana were evaluated (Figure 1.11.5).

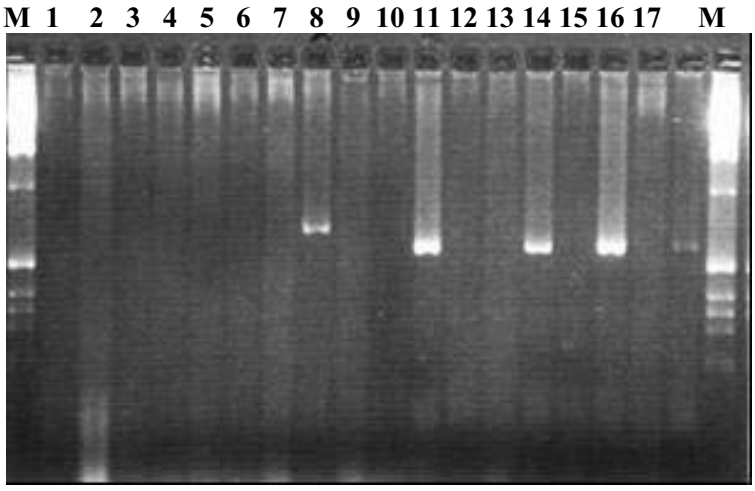


Figure 1.11.3. Amplified fragments of 0.6 and 0.7 kbp obtained with primers FSD-F/R from healthy and diseased plants of cassava varieties Manzana and ICA Catumare. Lanes 1–4 = amplified DNA from healthy tissue of variety Manzana; lanes 5–8 = infected tissue from variety Manzana; lanes 9–12 = amplified DNA from healthy tissue of variety ICA Catumare; lanes 13–16 = infected tissue from variety ICA Catumare; lane 17 = negative control; M = molecular weight marker of 1 kbp.

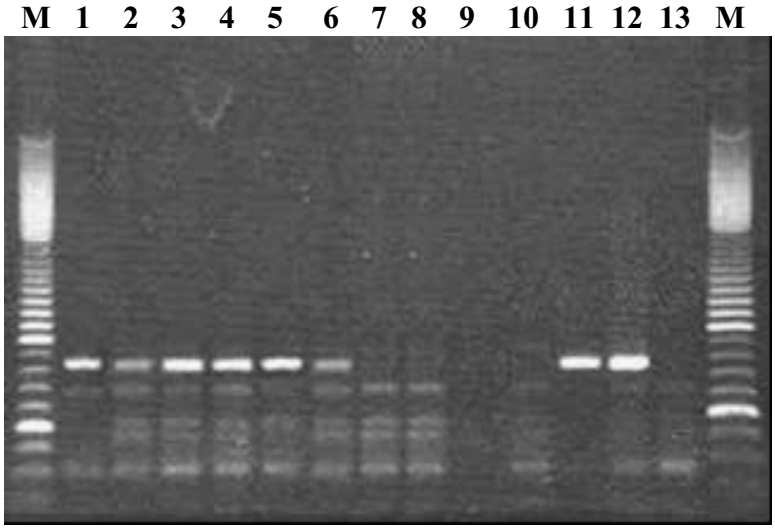


Figure 1.11.4. PCR amplification of one fragment of about 0.78 kbp with nested PCR, using primers P1/P7 and FSD-F/R. Lanes 1–10 = cassava frogskin disease phytoplasm (Cfdp) isolates (see Table 1.11.1); lanes 11 and 12, respectively = positive controls of Sqmp (*Solanum quitoense* machorreo phytoplasma) and Ccp (coffee crispiness phytoplasma); lane 13 = negative control; M = molecular weight marker of 1 kbp.

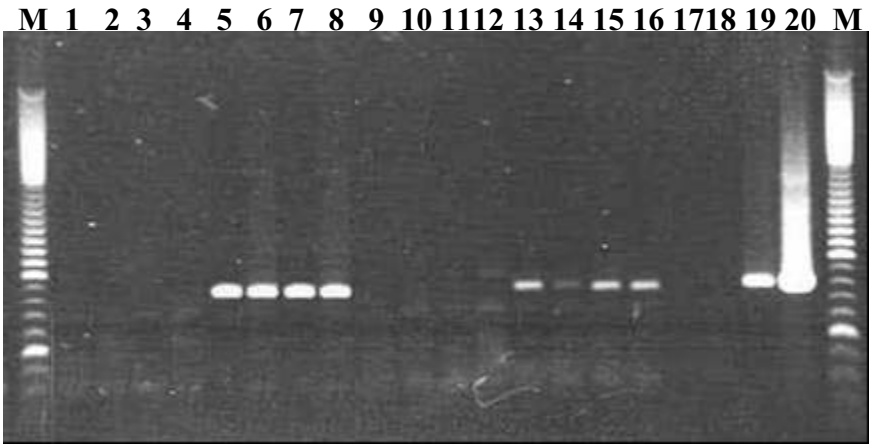


Figure 1.11. 5. Amplification of a fragment of 0.78 kbp with nested PCR, using primers P1/P7 and FSD-F/R, from healthy and diseased plants of varieties Manzana and ICA Catumare. Lanes 1–4 = healthy tissue of variety Manzana; lanes 5–8 = infected tissue; lanes 9–12 = healthy tissue from variety ICA Catumare; lanes 13–16 = infected tissue; lane 17 = negative control; M = molecular weight marker of 1 kbp.

Activity 1.12. Detecting phytoplasmas in cassava infected by cassava frogskin disease (CFSD), using nested PCR

Contributors: E. Álvarez, J. F. Mejía, G. Llano, and J. Loke

Highlight:

∉ FSD-specific primers proved highly specific and sensitive for detecting the phytoplasma. The PCR assay designed provided an effective alternative to conventional tests.

Rationale

Cassava frogskin disease (CFSD) attacks cassava roots, causing increasing numbers of deep lesions that eventually deform the roots. Despite its economic significance, the disease's causal agent has remained unknown for many years. Recently, CFSD has been reported with increasing frequency in Colombia, Brazil, and Venezuela. In Colombia, for example, incidence of up to 70% has been recorded in commercial fields in the production areas of Valle del Cauca, Cauca, Meta, and the North Coast. To develop appropriate management strategies for controlling the disease, the pathogen must be identified. Preliminary studies evidenced the existence of an association between CFSD and phytoplasmas. To confirm these results, molecular tools will be applied to detect phytoplasmas in CFSD-infected roots, leaf midribs, petioles, and peduncles in the different varieties and genotypes of cassava found in various regions of Colombia.

Materials and Methods

Phytoplasma sources: We took samples of leaf, stem, and root tissues from 39 genotypes of cassava (*Manihot esculenta* Crantz) grown in the field and greenhouse and naturally infected by CFSD. The plants were collected from 2002 to 2004 in three areas of Colombia—North Coast, Valle del Cauca, and Cauca—where the incidence of CFSD is high.

We included as negative checks cassava plants obtained *in vitro* through meristem culture. Positive checks were samples of periwinkle (*Catharanthus roseus* (L.) G. Don) that clearly showed typical symptoms induced by phytoplasmas—internode stunting or a clumping form of stunting in terminal buds.

Other checks included DNA from coffee crispiness phytoplasma (Ccp; GenBank Accession Number AY525125), facilitated by the National Coffee Research Center (CENICAFE, Colombia) and used as reference for the 16SrIII group (X-disease group); remolacha phytoplasma (Rp; 16SrIII group) and pimenton clover proliferation phytoplasma (Pcpp; 16SrVI group), both facilitated by the National Institute for Agricultural and Food Research, Madrid, Spain; and lethal wilt oil palm phytoplasma (Lwop) (GenBank Accession Number AY739024)

DNA extraction: DNA was extracted, following the protocol of Gilberston et al.(Gilbertson *et al.*, 1991, J Gen Virol 72:2843–2848.), from root, stem, and leaf petiole and nervure tissues sampled from plants in each geographical area.

PCR amplification: Three pairs of universal primers—**P1/P7** (Smart *et al*., 1996, Appl Envir Microbiol 62: 2988-2993.), **R16mF2/R1** (Gundersen and Lee, 1996, Phytopath Medit 35:114–151); Schneider *et al.*, 1993, J Gen Microbiol 139: 519–527.), and **R16F2n/R2** (Gundersen and Lee, 1996, Phytopath. Med 35:114-151)—were used in nested PCR to amplify the region of the genes 16S rRNA and 23S rRNA. The amplified products of P1/P7 and R16mF2/R1 were diluted at 1:50 with sterilized and distilled water for use as DNA mold in 1-µL quantities with primers R16F2n/R2.

Each reaction was put in 0.2-mL tubes carrying a volume of 25 µL, using final concentrations of 100 ng of DNA, 1X buffer, 3 mM $MgCl_2$, 1 U *Taq* polymerase (Promega, Madison, WI), 0.8 mM dNTPs (Invitrogen Life Technologies, Carlsbad, CA), and 0.1 µM of each primer (Operon Technologies, Inc., Alameda, CA).

For primers P1/P7, 35 cycles were carried out in a PTC-100 thermal cycler with a hot cover unit (MJ Research, Inc., Waltham, MA), following these conditions: 30 s (90 s for the first cycle) of denaturation at 94 °C, annealing for 50 s at 55 °C, and extension of the primer for 80 s (10 min in the final cycle) at 72 °C. Primers R16mF2/R16mR1 were amplified with 28 cycles, using the same conditions. The primer pair R16F2n/R16R2 was evaluated in a similar manner, but with an annealing temperature of 50 °C.

The PCR products were visualized in a 1.5% agarose gel, stained with 0.75 µg/mL ethidium bromide, and analyzed in a Stratagene Eagle Eye® II video system (La Jolla, CA).

PCR amplification with specific group primers: The DNA amplified with primers R16F2/R2 (16) was diluted at 1:50 and used as sample for re-amplification in nested PCR, using primers R16(III)F2/R16(III)R1 (16), designed specifically for the 16SrIII group of phytoplasmas (X-disease group), using the same PCR conditions as mentioned above, carrying out 35 cycles of 1 min (2 min for the first cycle) of denaturation at 94 °C, annealing for 2 min at 50 °C, and extension of the primer for 3 min (10 min in the final cycle) at 72 °C.

Results and Discussion

Detecting phytoplasmas associated with CFSD: Infected cassava and periwinkle plants exhibited symptoms typical of witches' broom in phytoplasmal infections. Nested PCRs primed by phytoplasma universal primer pair R16F2n/R2 resulted in the amplification of 1.2-kb DNA fragments of the 16S ribosomal DNA, indicating that the symptomatic cassava and periwinkle plants were infected by phytoplasmas. Phytoplasmas were detected in 35 of the 39 cassava varieties tested, exhibiting symptoms of CFSD, representing 89% of amplification (Table 1.12.1). The specific group primer products indicated

that the diseased cassava plants were infected by strains of a phytoplasma belonging to the 16SrIII group (X-disease group). The presence of a 16SrIII-group phytoplasma in all DNA samples was verified by nested PCR assays primed by primer pair R16(III)F2/R16(III)R1, which yielded an amplified product of about 0.8 kb. No amplified products were observed for Pcpp, Lwop, and periwinkle, which were used as controls.

Table 1.12.1. List of DNA fragments obtained from tissue samples from 39 varieties infected with cassava frogskin disease. The samples were amplified by direct and nested PCR, using universal primers and primers specific for phytoplasmas.

Sample code number	Variety number	Genotype	Site[a]	Tissue[b]	PCR[c]	Primers[d]
Y1	1	CM 6740-7	VC	LmP/R[e]	+/+	A–C
Y2	2	CIAT Parrita	VC	LmP/S/R	+/+/+	B
Y3	3	ICA Catumare	VC	LmP/R[e]	+/+	B–C
Y4	4	Manzana	VC	LmP/R[e]	+/+	B–C
Y5	5	M Bra 383	VC	LmP/R	+/+	B–C
Y6	6	CM 849-1	VC	LmP/R	+/+	B–C
Y7	7	CM 5460-10	VC	LmP	+/+	C
Y8	8	CM 2177-2	VC	LmP/R	+/+	B–C
Y9	9	CM 4919-1	VC	LmP/R	+/+	B–C
Y10	10	CM 3306-9	VC	LmP[e]	+	B–C
Y11	11	CM 3306-19	VC	LmP[e]	+	B–C
Y12	12	M Bra 856-54	VC	LmP[e]	+	B–C
Y13	13	M Per 335	VC	R	+	C
Y14	14	M Bra 856	C	LmP/R	+/+	C
Y15	15	SM 909-25	VC	LmP/R[e]	+/+	C
Y16	16	CG 6119-5	VC	LmP/R	+/+	C
Y17	17	M Col 2063	VC	LmP[e]	+	A–B–C
Y18	18	ICA Nataima	VC	LmP/R	-/+	C
Y19	19	SM 1201-5	VC	LmP	-	C
Y20	20	GM 228-14	VC	LmP	-	C
Y21	21	CM 9582-64	VC	LmP/R	+/+	A–B–C
Y22	22	CM 9582-65	VC	LmP/R	+/+	A–B–C
Y23	23	CM 9582-24	VC	LmP/R	+/+	A–B–C
Y24	24	M CR 81	VC	LmP/R	+/+	A–B–C
Y25	25	Venezolana	S	R	+	A–B–C
Y26	26	M Per 16	C	LmP/R	+/+	C
Y27	27	M Col 634	C	LmP/R	+/+	C
Y28	28	M Bra 829	C	LmP/R	+/+	C
Y29	29	SM 1219-9	VC	LmP/R[e]	+/+	A–B–C
Y30	30	M Chn 2	C	LmP/R	-/-	C
Y31	31	HMC-1	C	LmP/R	+/+	C
Y32	32	M Arg 2	C	LmP/R	-/-	C
Y33	33	M Bra 325	C	LmP/R	+/+	C
Y34	34	M Bra 839	C	LmP/R	+/+	C
Y35	35	M Col 1178	C	LmP/R	+/+	C
Y36	36	M Col 1468	C	LmP/R	+/+	C
Y37	37	M Cub 74	C	LmP/R	-/+	C
Y38	38	M Bra 886	C	LmP/R	+/+	C
Y39	39	M Bra 882	C	LmP/R	+/+	C
Y40	5	M Bra 383	C	LmP/R	+/+	B
Y41	3	ICA Catumare	Q	LmP/R	-/-	B–C
Y42	4	Manzana	Q	LmP/R	-/-	B–C

a. VC = Department of Valle del Cauca; C = Cauca; S = Sucre; Q = Quindío.
b. LmP = leaf midrib and petioles; R = roots; S = stems.
c. + = amplification positive for phytoplasma; - = amplification negative for phytoplasma.
d. Primers used for amplification were A = P1/P7–R16F2N/R16R2; B = R16mF2/R16mR1–R16F2N/R16R2; C = R16F2/R16R2–R16(III)F2/R16(III)R1.
e. Also showing foliar symptoms of chlorosis and deformed leaf blades.

Activity 1.13. Characterization of avirulence and resistance genes in the rice blast pathosystem

Contributors: D. Pulgarin and F. Correa

Highlight:

- The blast resistance genes present in 211 commercially grown Latin American rice cultivars were identified and nine groups of potential sources of complementary resistance genes were defined for their use in a breeding program aiming at developing commercial rice cultivars combining desired agronomic traits and blast resistance

Rationale

Rice blast is the most destructive disease of the crop in the world. Blast resistance in commercially released cultivars has not been durable, due to the lack of knowledge on the genetic constitution for blast resistance in the cultivars and the great variability of the blast pathogen. The blast resistance genes present in commercial rice varieties from Latin America are not known. One of the reasons for this situation includes the lack for many years of a differential system for the identification of the resistance genes. The main objective of this study was to identify blast resistance genes in 211 commercial rice cultivars from different Latin American countries following a differential system based on the gene-for-gene relationship between rice resistance genes and avirulence genes in the blast pathogen. The resistance genes were inferred based on the reaction patterns of monogenic differentials against 28 blast isolates from Colombia. The varieties were classified in nine complementary resistance groups, which can be used in genetic crosses aiming at combining complementary blast resistance genes for developing rice cultivars with desired agronomic traits and durable blast resistance. The information produced in this study is very important in relation to rice breeding programs that need to use resistant donors with desired agronomic traits and blast resistance.

Materials and Methods

A group of 40 international blast differentials (Table 1.13.1) with known blast resistance genes was used for the inoculation of blast isolates collected from infected samples and for the identification of avirulence genes present in the isolates. A total of 211 commercial rice cultivars from Latin America were inoculated and evaluated with blast isolates under greenhouse conditions for the identification of their blast resistance genes. Cultivars were selected from: Argentina (3), Bolivia (7), Brazil (30), Chile (2), Colombia (29), Costa Rica (12), Cuba (19), Ecuador (6), Guatemala (10), Guyana (1), Honduras (2), Mexico (17), Nicaragua (3), Panama (10), Paraguay (3), Peru (21), Dominican Republic (9), Salvador (5), Surinam (2), Uruguay (9), USA (2), and Venezuela (9).

One hundred and twenty blast isolates were obtained from infected samples collected from the different Latin American rice cultivars and breeding lines planted at the Santa Rosa, Meta experiment station in 2004. Monosporic isoloates were obtained in the pathology laboratory of the rice project and stored at –20C after growing them on filter paper.

Blast differentials were grown in 14 inches pots and inoculated with a spore suspension of 5×10^5 of each blast isolate. Plants were inoculated 21 days after sowing or at the third leaf stage in two replications with ten plants each. After inoculation, plants were incubated in plastic chambers with high relative humidity for 15 days allowing reinfection of the plants after the first cycle of reproduction of the pathogen. Plants were evaluated for the lesion type and leaf area affected exhibited after 15 days of incubation. Lesion types were scored from 1 to 4 were 1-2 were resistant types, 3 an intermediate type and lesion 4 as susceptible. Disease leaf area was scored from 1 to 100%. Commercial cultivars from Latin America were

inoculated with 28 blast isolates carrying between 1 to 14 avirulence genes determined on the inoculations on the differential set. Inoculation, incubation and evaluations were performed as for the differential set of cultivars.

Results and Discussion

Inoculation of blast isolates on the differential set of rice cultivars with known blast resistance genes allowed the selection of 28 blast isolates carrying between 1 to 14 avirulecne genes (Table 1.13.2). A resistant reaction observed after the inoculation of a rice differential carrying a known resistance gene indicates the presence of the corresponding avirulence gene in the blast isolate. These 28 blast isolates were used to inoculate the 211 commercial rice cultivars to infer the potential resistance genes present in their genetic constitution. A resistant reaction observed after the inoculation of a rice cultivar with an isolate carrying a known avirulence gene indicates the presence of the corresponding resistant gene in the cultivar.

As an example, Table 1.13.3 shows the possible resistant genes present in the cultivar ANAYANSI determined after the inoculation of the cultivar with the 28 blast isolates.
The compatible isolates A24, A19, A28, A20, A22, A5, and A17 (in red color) indicate that the resistant genes corresponding to the avirulence genes present in those 7 isolates can not be present in the cultivar ANAYANSI, otherwise the reaction would have been resistant. The resistant reaction observed for other isolates carrying similar avirulence genes (blue color) present in the 7 compatible isolates, cannot be due to corresponding resistance genes, but to other resistance genes. As a result, it can be concluded that the resistant reaction observed for those isolates exhibiting an incompatible reaction with the cultivar ANAYANSI should be the result on the interaction of the other avirulence genes present in the incompatible isolates as defined in Table 1.13.3. Based on this analysis, the resistance genes present in this cultivar were inferred as Pi-i, Pi-k, Pi-k^s, Pi-sh, Pi-t, Pi-ta^2, and Pi-z. Table 1.13.4 gives examples of 21 commercial rice cultivars from different Latin American countries indicating the diversity observed in terms of the potential number of blast resistant genes they carry, with 0 to all the 21 resistant genes present in the differentials. Further genetic studies including allelism tests will be developed to corroborate the presence of those genes in those cultivars.

Analysis of the frequency of the avirulence genes present among the 28 blast isolates used in this study were between 0.04 (Avr Pi-sh) and 0.96 (Avr Pi-9), indicating that most blast isolates have lost the avirulence gene for the resistance gene Pi-sh, while most still conserve the avirulence gene for the resistance gene Pi-9. This suggests that the resistant gene Pi-sh has not a valuable use as an individual gene and would have to be used in combination with other resistance genes. On the other hand, the high frequency observed for the avirulence gene Avr Pi-9, indicates that the Pi-9 gene could even be used as a single gene, although using it in combination with other blast resistance genes would ensure a longer life of the gene before the pathogen mutates to a compatible reaction. It should be noted however that given that one isolate (A27) exhibited a compatible reaction with the Pi-9 gene (Table 1.13.2), other blast resistant genes effective to this isolate should be combined with the Pi-9 gene (Table 1.13.2).

Analysis of the candidate blast resistant genes present in the 211 Latin American rice cultivars studied show in general a high negative correlation with the frequency of avirulence genes present in the pathogen population studied. A low frequency observed for an avirulence gene is associated with a high frequency of the corresponding resistance gene in the cultivars (Table 1.13.5 and Table 1.13.6). The frequency observed for Avr Pi-sh (0.04) is associated with a high frequency (0.84) of the resistant gene Pi-sh in the 211 commercial cultivars. On the other hand, the high frequency observed for the Avr Pi-9 (0.96) was associated with a low frequency (0.06) observed for the presence of the gene in the 211 cultivars (Table 1.13.5 and Table 1.13.6). For breeding purposes, those genes with a low frequency of use

(Table 1.13.6) would be of higher value than those genes that have been widely exposed to blast populations.

The 211 Latin American rice cultivars were classified into nine variety groups (Figure 1.13.1) based on the presence of 21 blast resistance genes analyzed in this study. These groups are considered complementary for different blast resistance genes and careful selection of different cultivars can provide useful combinations of resistance genes, which can confer durable blast resistance. As an example, Table 1.13.7 shows the frequency observed for three different blast resistant genes in three different variety groups an the complementarities among them to select rice cultivars for designing crosses aiming at combining the different resistance genes (Pi-1, Pi-2, Pi-33). While the three genes are in high frequency in group 3, the genes Pi-1 and Pi-33 are high in group 6 and gene Pi-2 is high in group 9. Crosses can be made between rice cultivars selected from groups 6 and 9 to combine the three genes, conferring a wider spectrum of resistance. Similar observations have been made for other genes and crosses have been designed with the purpose of pyramiding complementary blast resistance genes.

Commercially grown rice cultivars are characterized by having desired agronomic traits such as high yield, good grain quality, good adaptation, good plant type, and in many cases resistance to several pests and diseases. Most rice cultivars grown in Latin America have traits highly desired by rice farmers, however they have lost their resistance to blast, and therefore many of them are not grown anymore. A short-term breeding strategy aiming at developing rice cultivars with desired agronomic traits and combined blast resistance can be achieved if appropriate crosses between commercially blast susceptible rice cultivars are made. The information produced in this study is very important in relation to rice blast resistance breeding involving commercially grown blast susceptible cultivars from Latin America. This study demonstrates the utility of the differential system in elucidating the genetic constitution for blast resistance of 211 Latin American rice cultivars. Differential varieties are a very useful tool to identify blast resistance genes in rice and to characterize the pathogenicity of the blast pathogen. Latin American rice cultivars have been classified in different groups with complementary resistance genes, which can be used for designing genetic crosses aiming at combining resistance genes that can confer durable blast resistance. To confirm the different blast resistance genes identified in the rice cultivars from Latin America, genetic analysis using different progenies derived from crosses with susceptible varieties, allelism tests, and molecular markers associated with blast resistance genes will be conducted.

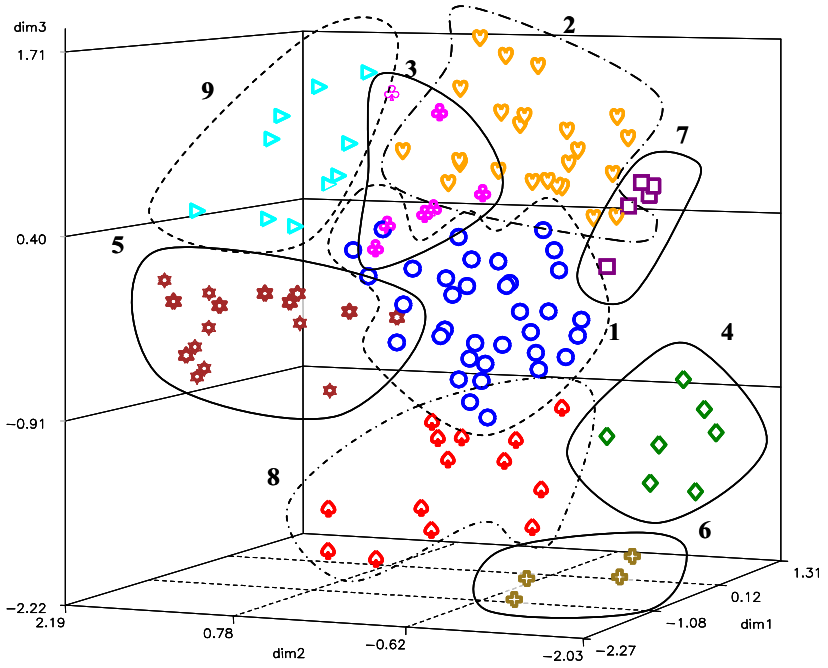


Figure 1.13.1 Spatial distribution of 211 Latin American rice cultivars in nine variety groups based on the presence of blast resistance genes

Table 1.13.1. Rice differentials with known blast resistance genes.

Number	Differential	Resistance Gene	Number	Differential	Resistance Gene
1	Fukunishiki	Pi-z, Pi-sh	21	Tsuyuake	Pi-k^{m}
2	Fujisaka 5	Pi-I, Pi-k^{s}	22	Nipponbare	Pi-sh
3	BL-1	Pi-b	23	Ou 244	Pi-z
4	BL-2	Pi-b	24	Ishikari Shiroke	Pi-I, Pi-k^{s}
5	Toride 1	Pi-z^{t}	25	Tetep	Pi-k^{h}
6	K3	Pi-k^{h}	26	IR 22	Pi-k, Pi-sh, Pi-ta^{2}
7	K 59	Pi-t	27	C 101 A 51	Pi-2
8	K60	Pi-k^{p}	28	C 101 LAC	Pi-1, Pi-33
9	C 104 LAC	Pi-1	29	C 101 PKT	Pi-4a
10	C 103 TTP	Pi-1	30	C 104 PKT	Pi-3
11	C 105 TTP-1	Pi-ta, Pi-4a	31	C 105 TTP-4	Pi-4b
12	F 128-1	Pi-ta^{2}	32	CT 13432-6	Pi-33
13	F 145-2	Pi-b	33	CT 13432 –68	Pi-1
14	Zenith	Pi-z, Pi-a	34	CT 13432-267	Pi-2
15	Pi No. 4	Pi-sh, Pi-ta^{2}	35	Dular	Pi-k^{a}
16	Rico 1	Pi-k^{s}	36	75-1-127	Pi-9
17	Norin 22	Pi-sh	37	K1(Francia)	Pi-ta
18	Nato	Pi-I	38	CT 13432-107	Pi-1, Pi-2, Pi-33
19	Shin 2	Pi-sh, Pi-k^{s}	39	Zenith (Brasil)	Pi-z, Pi-a
20	Kanto 51	Pi-k	40	Fanny	None

Table 1.13.2. Predicted avirulence genes present in 28 *Pyricularia grisea* isolates.

Isolates	Blast Resistance Genes																				Avr Gene No.
	Pib	**Pii**	**Pik**	**Pik^{a}**	**Pik^{h}**	**Pik^{m}**	**Pik^{p}**	**Pik^{s}**	**Pish**	**Pi-t**	**Pita**	**Pta^{2}**	**Pi-z**	**Piz^{t}**	**Pi1**	**Pi2**	**Pi3**	**Pi9**	**Pi33**	**Pi4a**	**Pi4b**	
A12. CT 13432-107 (14-1)																		1				1
A14. CT 13432-34 (25-1)																		1				1
A21. CL 00-2-1-38																		1				1
A 10. CT 13432-246 (13-1)										1								1				2
A 13. CT 13432-107 (25-1)										1								1				2
A08. O.Yacu 9-17-1													1					1	1			3
A01. Fanny 54													1	1				1	1			4
A24. CL 1-6-13														1		1	1	1				4
A25. CL 00-6-1-7				1													1	1	1			4
A02. O. Caribe 8-17-1			1	1	1	1												1				5
A19. CL 00-4-1-25					1	1									1			1	1			5
A23. CL 1-1-41				1	1	1									1			1				5
A11. Isolinea 6-7-1	1						1						1	1		1		1				6
A16. CT 13432-107 (12-1)			1	1	1							1			1			1				6
A28. CR 1821 (1-1)				1							1			1		1		1			1	6
A06. FL 00440-30P-2-2P-M			1	1	1	1				1					1			1				7
A09. FED 2000-5-1				1	1	1				1					1			1			1	7
A18. CL 00-4-1-24		1		1			1						1				1	1	1			7
A07. FED 50-24-1			1	1	1	1				1	1				1			1				8
A20. CL 3-6-25				1			1				1			1		1	1	1		1	1	9
A22. CL 00-1-1-36				1	1	1								1		1	1	1	1		1	9
A15. COL XXI		1		1	1	1	1						1		1		1	1	1			10
A26. Irga 409	1			1			1				1	1	1			1	1	1	1		1	11
A03. Metica 1-33-18			1	1	1	1							1		1	1	1	1	1	1	1	12
A05. Cica 9-15	1			1	1	1	1				1			1	1		1	1	1	1	1	13
A17. CL 00-3-1-29				1	1	1	1				1			1	1	1	1	1	1	1	1	13
A27. Cimarron 1-2	1			1	1		1	1	1	1			1	1	1	1	1		1			13
A04. Cica 9-37-1		1		1	1	1	1	1			1		1		1		1	1	1	1	1	14

1= incompatible reaction indicating the presence of the corresponding avirulence gene in the pathogen.

Table 1.13.3. Predicted resistance genes present in the commercial rice cultivar ANAYANSI (Pi-i, Pi-k, Pi-k^s, Pi-sh, Pi-t, Pi-ta^2, Pi-z) base susceptible reaction to isolates A24, A19, A28, A20, A22, A05, A17.

	Blast Resistance Genes																		
Isolates	**Pib**	**Pii**	**Pik**	**Pik^a**	**Pik^h**	**Pik^m**	**Pik^p**	**Pik^s**	**Pish**	**Pi-t**	**Pita**	**Pta^2**	**Pi-z**	**Piz^t**	**Pi1**	**Pi2**	**Pi3**	**Pi9**	**Pi33**	**Pi**
A12. CT 13432-107 (14-1)																		1		
A14. CT 13432-34 (25-1)																		1		
A21. CL 00-2-1-38																		1		
A 10. CT 13432-246 (13-1)										**1**								1		
A 13. CT 13432-107 (25-1)										**1**								1		
A08. O.Yacu 9-17-1													**1**					1	1	
A01. Fanny 54													**1**	1				1	1	
A24. CL 1-6-13														1		1	1	1		
A25. CL 00-6-1-7				1													1	1	1	
A02. O. Caribe 8-17-1			**1**	1	1	1												1		
A19. CL 00-4-1-25					1	1									1			1	1	
A23. CL 1-1-41				1	1	1									1			1		
A11. Isolinea 6-7-1	1						1						**1**	1		1		1		
A16. CT 13432-107 (12-1)			**1**	1	1							**1**			1			1		
A28. CR 1821 (1-1)				1							1			1		1		1		
A06. FL 00440-30P-2-2P-M			**1**	1	1	1				**1**					1			1		
A09. FED 2000-5-1				1	1	1				**1**					1			1		
A18. CL 00-4-1-24		**1**		1			1						**1**				1	1	1	
A07. FED 50-24-1			**1**	1	1	1				**1**	1				1			1		
A20. CL 3-6-25				1			1				1			1		1	1	1		
A22. CL 00-1-1-36				1	1	1								1		1	1	1	1	
A15. COL XXI		**1**		1	1	1	1						**1**		1		1	1	1	
A26. Irga 409	1			1			1				1	**1**	**1**			1	1	1	1	
A03. Metica 1-33-18			**1**	1	1	1							**1**		1	1	1	1	1	
A05. Cica 9-15	1			1	1	1	1				1			1	1		1	1	1	
A17. CL 00-3-1-29				1	1	1	1				1			1	1	1	1	1	1	
A27. Cimarron 1-2	1			1	1		1	**1**	**1**	**1**			**1**	1	1	1	1		1	
A04. Cica 9-37-1		**1**		1	1	1	1	**1**			1		**1**		1		1	1	1	

Red: compatible isolates predict susceptibility of resistance genes matching corresponding avirulence genes present in the islate; **blue**: inferred susceptibility of resistance gene; **black:** inferred resistance gene based on incompatible reactions with isolates carrying corresponding avirulence genes

Table 1.13.4. Predicted blast resistance genes present in 21 Latin American commercial rice cultivars based on greenhouse inoculations with rice blast isolates carrying corresponding avirulence genes

Commercial Cultivar	Country	Blast Resistance Genes																				
		Pi-b	Pi-i	Pi-k	Pi-k^a	Pi-k^h	Pi-k^m	Pi-k^p	Pi-k^s	Pi-sh	Pi-t	Pi-ta	Pi-ta^2	Pi-z	Pi-z^t	Pi-1	Pi-2	Pi-3	Pi-9	Pi-33	Pi-4a	Pi-4b	No.
P. industrial	**Chile**																						0
BR-Irga 412	**Brazil**			X																			1
CR 750	**Costa Rica**											X									X		2
Oryzica 1	**Colombia**	X								X			X										3
Juma 61	**R. Dominicana**			X						X			X				X						4
Linea 2	**Colombia**	X		X					X	X			X										5
Iniap 12	**Ecuador**	X	X						X	X				X						X			6
CR 1113	**Costa Rica**		X	X		X	X		X	X						X							7
Iacuba 16	**Cuba**		X	X		X	X		X	X			X			X							8
Palmar PA	**Argentina**	X	X					X	X	X							X	X			X	X	9
Damaris	**Panama**	X	X	X					X	X	X		X	X		X				X			10
Fedearroz V1	**Colombia**	X	X	X				X	X	X		X	X				X	X			X		11
Colombia 21	**Colombia**	X	X	X				X	X	X		X			X		X	X		X	X		12
Icta Virginia	**Guatemala**		X	X	X	X	X		X	X		X	X			X		X			X	X	13
Inia Zapata	**Uruguay**	X	X	X		X	X	X	X	X			X	X		X		X		X	X		14
Panacu	**Bolivia**	X	X	X		X	X	X	X	X	X		X	X		X		X		X	X		15
Icta	**Guatemala**	X	X	X	X	X	X	X	X	X		X	X			X	X	X			X	X	16
Colomgua																							
Altamira 7	**Nicaragua**	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X			X	X	18
Fedearroz 50	**Colombia**	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X		X	X	X	19
Cuyamel 3820	**Honduras**	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	20
Triunfo	**Brazil**	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	21

Table 1.13.5. Frequency of avirulence genes in 28 blast isolates selected to screen commercial rice cultivars from Latin America.

Avirulence Gene	Isolate No.	Frequncy	Avirulence Gene	Isolate No.	Frequency
Avr Pi-sh	1	0.04	*Avr Pi-z*	9	0.32
Avr Pi-k^{s}	2	0.07	Avr Pi-z^{t}	9	0.32
Avr Pi-ta^{2}	2	0.07	Avr Pi-2	9	0.32
Avr Pi-i	3	0.11	Avr Pi-4b	9	0.32
Avr Pi-b	4	0.14	Avr Pi-k^{m}	12	0.43
Avr Pi-k	5	0.18	Avr Pi-1	12	0.43
Avr Pi-4^{a}	5	0.18	Avr Pi-3	12	0.43
Avr Pi-t	6	0.21	Avr Pi-33	13	0.48
Avr Pi-ta	7	0.25	Avr Pi-k^{h}	14	0.50
Avr Pi-k^{p}	9	0.32	Avr Pi-k^{a}	18	0.64

Table 1.13.6. Frequency of blast resistance genes in 211 commercial rice cultivars from Latin America.

Resistance Gene	Cultivars No.	Frequency	Resistance Gene	Cultivar No.	Frequency
Pi-9	12	0.06	***Pi-4b***	69	0.33
Pi-k^{a}	35	0.17	**Pi-3**	83	0.39
Pi-t	46	0.22	**Pi-ta**	94	0.45
Pi-z^{t}	48	0.23	**Pi-k**	99	0.47
Pi-k^{h}	55	0.26	**Pi-b**	112	0.53
Pi-k^{m}	55	0.26	**Pi-i**	117	0.55
Pi-z	62	0.29	**Pi-4a**	125	0.59
Pi-k^{p}	64	0.30	**Pi-ta^{2}**	137	0.65
Pi-1	64	0.30	**Pi-k^{s}**	158	0.75
Pi-33	64	0.30	**Pi-sh**	178	0.84
Pi-2	68	0.32			

Table 1.13.7. Example of complementary resistance genes between complementary groups of Latin American rice cultivars.

Complementary Group	Resistance Gene	Frequency
Group 3	Pi-1	1.00
	Pi-2	1.00
	Pi-33	0.82
Group 6	Pi-1	1.00
	Pi-2	0.00
	Pi-33	1.00
Group 9	Pi-1	0.10
	Pi-2	1.00
	Pi-33	0.10

Activity 1.14. Effects of endophytic bacteria on plant growth and development

Contributors: P. Fory, S. Kelemu, J. Ricaurte, R. Garcia and I. Rao

Highlights:

- Demonstrated that through tissue culture and spraying antibiotics (cefotaxime and vancomycin) we could eliminate endophytic bacteria in Brachiaria, which is necessary step to determine their fixing properties.
- Showed that through introduction of bacteria isolated from a Brachiaria hybrid (CIAT 36062) the Brachiaria hybrid cv. Mulato (CIAT 36061) exhibited improved growth (more litters and root development) relative to the control (indigenous bacteria only).
- Developed a specific primer useful to detect endophytic bacteria associated with Brachiaria using one step PCR instead of nested PCR.

Rationale

In both managed and natural ecosystems, plant-associated bacteria play key roles in host adaptation to changing environments. These interactions between plants and beneficial bacteria can have significant effect on general plant health and soil quality. Associative nitrogen-fixing bacteria may provide benefits to their hosts as nitrogen biofertilizers and plant growth promoters. Several endophytic bacteria have been reported to enhance growth and improve plant health in general (Sharma and Novak, 1998, Can. J. Microbiol. 44: 528-536; Stoltzfus *et al*., 1998, Plant Soil 194:25-36). Many plant-growth-promoting bacteria (PGPB) that include a diverse group of soil bacteria are thought to stimulate plant growth by various mechanisms such as plant protection against pathogens, providing plants with fixed nitrogen, plant hormones, or solubilized iron from the soil.

Brachiaria grasses of African savannahs have supported millions of African herbivores over thousands of years. Some of these *Brachiaria* species have many desirable agronomic traits. For example, they are persistent and can grow in a variety of habitats ranging from waterlogged areas to semi-desert. These grasses that are often grown under low-input conditions are likely to harbour unique populations of nitrogen-fixing or plant growth promoting bacteria. The aim of our study is to examine the effects of endophytic bacteria that were isolated from species of *Brachiaria* on plant development.

Materials and Methods

Bacterial inoculum preparation: Three endophytic bacterial isolates 01-36062-R2, 02-36062-H4, and 03-36062-V2 that were originally isolated from *Brachiaria* CIAT 36062 in roots, leaves and stems, respectively, and that tested positive for sequences of the *nif*H gene (the gene that encodes nitrogenase reductase) are maintained at -80°C in 20% glycerol. Bacterial cells were removed from each of the stored samples, plated on nutrient agar medium (Difco, Detroit, MI) and incubated for 24 h at 28°C. The cells from each of the bacterial strains were collected from the plates, suspended in sterile distilled water and adjusted to a concentration of optical density (OD_{600}) = 1.0 with a spectrophotometer.

Plant inoculation: Twenty tillers of about a month old were prepared from a single mother plant of *Brachiaria* hybrid CIAT 36061 (cv. Mulato), their roots washed with sterile distilled water and made ready for inoculations. The roots of ten of these tillers were immersed in a beaker containing a mixture of equal volumes (50-ml each) of the three strains of endophytic bacterial suspension described above. The remaining ten plants were immersed in a beaker containing the same volume of sterile distilled water. All plants were kept immersed for 48 hours, after which they were removed and rinsed 3 times with sterile

distilled water. They were then each transplanted to pots containing sterile sand (95%) and soil (5%) and maintained in the greenhouse under natural day light and at temperatures between 19 and 30°C. No nutrients were applied.

Plant evaluations: Sixty-five days after inoculations the following measurements were taken in control and treated plants: 1) plant growth and development such as plant height, number of tillers, number of leaves, leaf area, 2) leaf chlorophyll content, 3) nitrogen content, and 4) soluble protein content in leaves.

Plant development and other measurements: Plant height was measured in centimeters from stem base to the highest part of the plant. Number of leaves per plant and the number of tillers were determined. Leaf area was determined in cm^2/plant and measured using a LI-300 leaf area meter (LI-COR, inc., Lincoln, NE). In addition, dry matter distribution among leaves, stems and roots was determined after drying each tissue separately in an oven at 70ºC for 48 hours. Leaf chlorophyll content was measured with a chlorophyll meter SPAD 502 (Minolta), taken across the third fully developed leaf as an average of 6 measurements. Soluble leaf protein was measured as described by Rao and Terry (Plant Physiol 90: 814-819). Nitrogen content in leaves and stems was determined using methods described by Salinas and García (1985, CIAT, Working document 83 p).

Bacterial population in the roots: Approximately 1 g of root sample was taken from each individual plant, surface sterilized (in 1% NaOCl solution for 2 min, in 70% ethanol for one min, then rinsed 3 times in sterile distilled water) and macerated in mortar and pestle in 1 ml of sterile distilled water. One hundred-εl of this macerated sample was taken and a dilution series performed. These were plated on nutrient agar medium and incubated for 24 h at 28°C to determine bacterial colony growth, and calculate the number of bacterial cell per gram of root weight.

Experimental design and statistical analysis: The experiment had two treatments (with and without artificial inoculations) each with 10 plants and arranged in a completely randomized design. Analysis of variance was determined using Statistics Analysis System (SAS⊇). A t-test was conducted.

Results and Discussion

Brachiaria hybrid CIAT 36061 had indigenous endophytic bacteria that are difficult to eliminate. Because of the difficulty to eliminate these indigenous bacteria, we set out to introduce three different strains of bacteria, originally isolated from *Brachiaria* hybrid CIAT 36062, into CIAT 36061, in addition to the indigenous bacteria that this hybrid already has. In general, the introduction of these bacteria had a positive effect on plant growth and development in the recipient plant CIAT 36061 (Figure 1.14.1). Figure 1.14.2 further demonstrates more tiller and root development in artificially inoculated CIAT 36061 plants than plants containing only indigenous endophytic bacteria.

Figure 1.14.1. *Brachiaria* hybrid CIAT 36061 plants with indigenous endophytic bacteria (1), and inoculated with a mixture of 3 bacterial strains 01-36062-R2, 02-36062-H4, and 03-36062-V2 (originally isolated from *Brachiaria* CIAT 36062) [2], 65 days after inoculations and maintained under greenhouse conditions with no nutrients.

In nitrogen- and other nutrient-deficient conditions, *Brachiaria* plants inoculated with the three bacterial strains had significantly higher average values in all evaluated parameters (with the exception of soluble proteins in leaves) than those control plants containing just indigenous bacteria (Table 1.14.1).

Table 1.14.1. Average values of various parameters evaluated for endophyte-inoculated and non-inoculated plants of CIAT 36061.

Parameters	Control	Inoculated
Plant height (cm)	103.9b[†]	115.6a**
Leaves/ plant	22.5b	36.9a***
Tillers/ plant	4.1b	7.4a***
Leaf area (cm^2/plant)	993.7b	1430a***
Chlorophyll SPAD	340.3b	433.7a***
Soluble Protein (σg/ cm^2 fresh leaf)	928.93a	1095.42[a]
Stem N content (%)	0.51b	0.67a**
Green leaf N content (%)	1.0 b	1.3a **
Dead leaf N content (%)	0.44b	0.66a**

[†]Each value is the mean of values from 10 plants. Data in each row followed by the same letter are not significantly different (P<0.05) according to t-test.

Figure 1.14.2. A *Brachiaria* hybrid CIAT 36061 with indigenous endophytic bacteria (1), and inoculated with a mixture of 3 bacterial strains 01-36062-R2, 02-36062-H4, and 03-36062-V2 (originally isolated from *Brachiaria* CIAT 36062) [2], 65 days after inoculations and maintained under greenhouse conditions with no nutrients. Note the difference between the artificially inoculated plant and the one with just indigenous bacteria, in the number of tillers and root growth and development.

Analysis of variance showed that the total biomass production (leaf, stem and root) collected from control Brachiaria CIAT 36061 plants was significantly ($P< 0.05$) less than that from inoculated ones (Figure 1.14.3). The data presented indicate that a close and beneficial interaction existed between the introduced as well as indigenous endophytic bacteria and *Brachiaria* hybrid CIAT 36061, resulting possibly in nitrogen fixation and enhancement of plant growth. Had we managed to eliminate the indigenous endophytic bacteria from control CIAT 36061 plants, the difference between bacteria-containing and control plants would probably have been even more dramatic.

A high correlation value ($r= 0.89$; $P<0.01$) was observed between leaf chlorophyll content and % nitrogen in leaves. Inoculated plants maintained a more profound green color and higher nitrogen content in their leaves than control plants. As expected, bacterial cells were isolated from both control plants containing indigenous bacteria and those inoculated with the 3 bacterial strains with values that are similar (6.56 $\log_{10}$ CFU/g of fresh root tissue of inoculated plants vs 6.53 $\log_{10}$ CFU/g of fresh root tissue of non-inoculated control plants). These endophytic bacterial population data are very similar to the natural endophyte concentrations in alfalfa, sweet corn, sugar beet, squash, cotton and potato, reported to vary between 2.0 and 6.0 $\log_{10}$ CFU/g of tissue (Kobayashi and Palumbo, 2000, *In* Bacon and White, eds. Microbial endophytes. Marcel Dekker, Inc., NY).

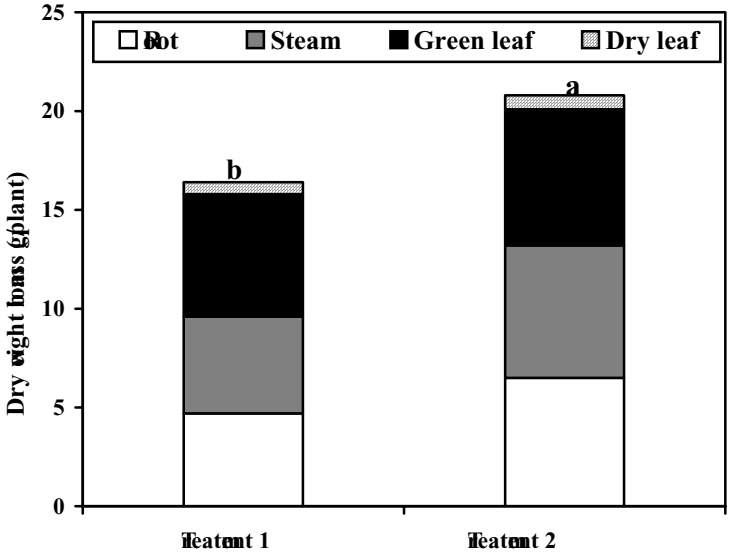


Figure 1.14.3. Total tissue biomass production in *Brachiaria* hybrid CIAT 36061 control plants with indigenous endophytic bacteria (treatment 1), and inoculated with a mixture of 3 bacterial strains 01-36062-R2, 02-36062-H4, and 03-36062-V2 (originally isolated from *Brachiaria* CIAT 36062) [treatment 2], 65 days after inoculations and maintained under greenhouse conditions with no nutrients. Values are average of 10 plants per treatment.

These preliminary data strongly suggest that endophytic bacteria have a direct beneficial effect on plant growth and development, and possibly on associated nitrogen fixation in *Brachiaria*. More work is needed to further verify these findings preferably after completely removing indigenous bacteria from species of *Brachiaria*.

Activity 1.15. Cloning and characterization of a nitrogen fixation gene (*nif*) sequences from a plant growth promoting bacterium associated with species of *Brachiaria*.

Contributors: P. Fory and S. Kelemu

Rationale

A number of prokaryotes are known to be involved in nitrogen fixation as well as enhancement of plant growth. Nif genes which encode the nitrogenase complex (encoded by approximately 20 different nif genes) and other enzymes involved in nitrogen fixation has consensus sequences identical from one nitrogen fixing bacteria to another, but while the structure of the nif genes is similar, the regulation of the nif genes varies between different diazotrophes (nitrogen fixing organisms).

Previously, we have reported the isolation of three strains of bacteria from *Brachiaria* hybrid CIAT 36062 (BR97-1371) from roots, leaves and stems that were designated 01-36062-R2, 02-36062-H4, and 03-36062-V2, respectively. Using nested PCR and three primers designed for the amplification of the *nifH* gene sequences, amplified products were generated with template DNA from these bacterial strains. We have also reported in 2003 that fatty acid analysis conducted on these 3 strains resulted in matching them

with various bacteria that are known to be nitrogen fixers and/or plant growth promoters (for example with *Flavimonas oryzihabitans*).

This study focused on strain 01-36062-R2. The fatty acid analysis data of the isolate 01-36062-R2 matched it with *Leclercia adecarboxylata*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*, at 0.879, 0.841, and 0.820 similarity index, respectively (IP-5 Annual Report 2003). *E. cloacae* has been described as one of the dominant endophytic bacteria isolated from citrus plants.

In this study we cloned and sequenced nested PCR amplified products using primers derived from nif-gene sequences. The objective of this work is to develop a specific primer that will allow us to screen (without using nested PCR) *Brachiaria* and other tropical plants-associated bacteria that contain *nif*-gene sequences.

Materials and Methods

Bacterial isolates: Bacterial strain isolated from roots of *Brachiaria* CIAT 36062 and designated 01-36062-R2 was used for this study. For evaluation of the various primers, strains isolated from stems and leaves of the same *Brachiaria* genotype, and designated 02-36062-H4 y 03-36062-V2, *Bradyrhizobium* species CIAT 2469 isolated from the legume Desmodium species, and a pathogenic bacterium *Xanthomonas campestris* pv. *graminis* (included as a negative control) were used.

Bacterial DNA extractions: DNA extraction was conducted using a modified protocol based on combinations of standard methods, which includes growing bacterial cells in liquid media LB (tryptone 10g, yeast extract 5g, NaCl 10g, 10 ml of 20% glucose in 1 L of distilled water), treatment of cells with a mixture of lysozyme (10 mg.ml in 25 mM Tris-Hcl, ph 8.0) and RNase A solution, and extraction of DNA with STEP (0.5% SDS, 50 mM Tris-HCl 7.5, 40 mM EDTA, proteinase K to a final concentration of 2mg/ml added just before use. The method involves cleaning with phenol-chloroform and chloroform/isoamyl alcohol and precipitation with ethanol. The quality of DNA was checked on 1 % agarose gel.

Nested PCR Amplification: Three primers were used, which were originally designed by Zehr and McReynolds (1989, Appl. Environ. Microbiol. 55: 2522-2526) and Ueda *et al*. (1995, J. Bacteriol. 177: 1414-1417), to amplify fragments of *nifH* genes. Amplification steps described by Widmer *et al* (1999, Applied and Environmental Microbiology 65:374-380) were adopted. The final product of the nested PCR amplification is about 370 bp in size.

Cloning of amplified DNA fragments: Amplified products were eluted from agarose gel using Wizard ® PCR Preps DNA Purification System (Promega) according to instructions supplied by the manufacturer. The purified fragments were ligated to the cloning vector pGEM-T Easy (Promega) and used to transform *E. coli* DH5Ŭusing standard procedures (Sambrook *et al*., 1989. Molecular Cloning: a laboratory manual. 2nd ed. Cold spring harbor laboratory, USA)

Plasmid extraction: Plasmids were extracted from transformed *E. coli* DH5Ŭcells using a Wizard® Plus Mini-preps DNA Purification System (Promega) using the protocol supplied by the manufacturer. To confirm whether the transformants contained the desired size of insert (approximately 370 bp), the plasmid DNA was digested to completion with the restriction enzyme *Eco*RI. The digested products were separated by electrophoresis on a 1% agarose gel (Bio-Rad, NJ), stained with ethidium bromide and photographed under UV light.

Amplification of DNA inserts for sequencing: PCR reactions (25-σl) contained 20 ng/σl plasmid DNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, primers T7 (5'-GTAATACGACTCACTATAGGGC-3')

and Sp6 (5' –TATTTAGGTGACACTATAG-3') each at 0.1 σM concentration, 0-5U Taq polymerase and amplified in a programmable thermal controller (MJ Research, Inc, MA) programmed with 35 cycles of a 30 sec (2 min for the first cycle) denaturation step at 94°C, annealing for 30 sec at 50°C, and primer extension for 1 min (4 min in the final cycle) at 72°C. Samples of amplified products were separated on a 2% agarose gel by electrophoresis for further confirmation of the expected size insert.

The ABI prism BigDye terminator Cycle sequencing kit was used to further prepare the samples for sequencing. Sequencing was conducted using ABI PRISM^{TM} 377 DNA sequencer. The sequence data were compared with sequences in databases using the program BLAST version 2.0 or 2.1 (http://www.ncbi.nlm.nih.gov/BLAST/-). The program compares nucleotide sequences to databases and calculates the statistical significance of matches.

Specific primer construction: Based on the sequence data, primers were designed using the program DNA-MAN (version 4:0), and synthesized by Integrated DNA Technologies, Inc. (Coralville, USA). These primers were tested on bacteria that are confirmed positive and negative controls: strains 01-36062-R2; 02-36062-H4 and 03-36062-V2, *Bradyrhizobium*, and *Xanthomonas campestris* pv. *graminis.*

Results and Discussion

Cloning and sequence analysis: A 371 bp nested PCR amplification product using template DNA isolated from the bacterial strain 01-36062-R2 (a strain isolated from *Brachiaria* CIAT 36062 and that tested positive for *nif*-gene sequences) was successfully cloned in the vector pGEM-T Easy (Promega). Figure 1.15.1 shows randomly picked transformants, the majority showing the desired size insert, with the exception of lanes 2 and 7.

The sequence analysis demonstrated the presence of *nif*H gene sequences in these sequenced clones. The deduced amino acid sequence showed a 97% similarity with 120 amino acids that correspond to the *nif*H gene sequence of *Klebsiella pnuemoniae*. These results are in agreement with the fatty acid analysis results of this bacterial strain that matched it with *Klebsiella pnuemoniae* at 0.84 similarity index. *Nif* genes that encode the nitrogenase complex and other enzymes involved in nitrogen fixation have consensus sequences identical in various nitrogen-fixing bacteria.

Klebsiella pneumoniae is a member of the Enterobacteriaceae that has the ability to fix nitrogen, and possesses a total of 20 *nif* genes that are clustered in a 24 kb region of the chromosome and responsible in nitrogenase synthesis and its regulation. Three of these genes, *nif*HDK, code for the three structural nitrogenase subunits. *K. pnuemoniae* has been reported as an endophytic bacterium associated with various plants and involved in nitrogen fixation, including maize (Chelius and Triplett, 2001, Microb. Ecol. 41: 252–263), wheat (Iniguez *et al*., 2004, Molecular Plant-Microbe Interactions 17: 1078–1085) and rice (Dong *et al*., 2003, Plant Soil 257:49-59).

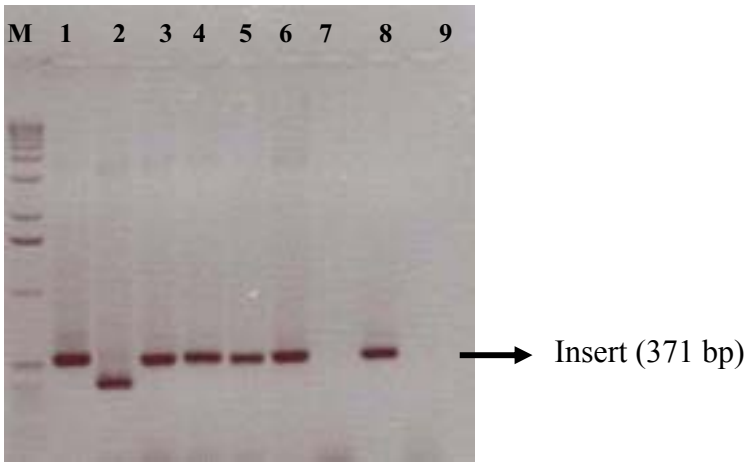


Figure 1.15.1. Amplified products of independent clones using template DNA of the bacterial strain 01-36062-R2 associated with *Brachiaria* CIAT 36062. Amplification conditions were as described in the materials and methods section. M = molecular marker. Lanes 1-8 inserts of clones, lane 9 = negative control. The clones that showed the expected 371 bp size insert were sequenced.

The consensus sequences obtained in this study are listed below as deduced amino acid sequences (**1**) and nucleotide sequences (**2**).

1.
GVIQADSTRLILHAKAQNTIMEMAAEVGSVEDLELEDVLQIGYGGVRCAESGGPE
PGVGCAGRGVITAINLEEEGAYVPDLDFVFYDVLGDVVCGGFAMPIRENKAQEIY
IVCSGEMMALYA

2.
5'-
TGGGTGTGATCCAAGCCGACTCCACGCGTTTGATCCTGCATGCGAAAGCGCAGA
CACCATTATGGAGATGGCCGCCGAAGTCGGCTCCGTCGAAGACCTGGAATTAGA
GACGTGCTGCAAATCGGTTACGGCGGCGTGCGCTGCGCGGAATCCGGTGGCCCG
AGCCAGGTGTGGGCTGTGCCGGTCGTGGCGTGATCACCGCGATTAACTTCCTCG
AGAAGAAGGCGCTTACGTGCCGGATCTGGATTTTGTTTTCTACGACGTGCTGGG
GACGTGGTATGCGGTGGTTTCGCCATGCCGATTCGTGAAAACAAAGCGCAGGAG
TCTACATCGTTTGCTCTGGCGAGATGATGGCCCTCTACGCA-3'

Specific primer development: In this study we developed specific primers that would allow us detect endophytic bacteria associated with species of *Brachiaria* just with one step PCR instead of nested PCR. Based on the consensus sequence listed above (in #2) and using the DNA-MAN program, 9 primers were designed and synthesized. Twenty combinations of these primers were tested on selected positive and negative control bacteria. Of these combinations, a pair of primers with sequences 5'-GTTTGATCCTGCATGCAAAAG-3' and 5'-AGAGCAAACGATGTAGATCTCCTG- 3', produced only one amplification product with a size of approximately 344 bp in bacteria that are used as positive

controls, where as negative controls resulted in no amplified products (Figure 1.15.2). This pair of primers will be tested on various bacteria associated with species of *Brachiaria* and other plants and that are suspected to fix nitrogen. We also want to use this pair of primers to directly detect these bacteria in tissues or soil samples.

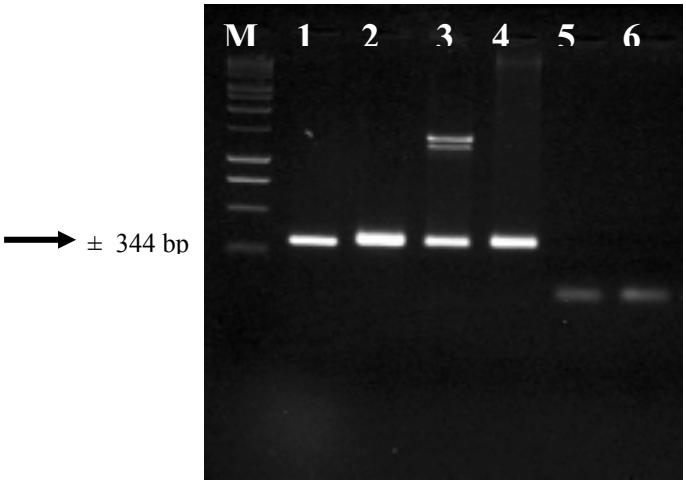


Figure 1.15.2. Specific amplifications of template DNA from lanes 1-4, positive control bacteria *Bradyrhizobium* sp., strains 01-36062-R2; 02-36062-H4 and 03-36062-V2 isolated from *Brachiaria*, respectively; lane 5, negative control *Xanthomonas campestris* pv. *graminis*; lane 6: negative control PCR reaction mixture; M, size marker. Primer combination used 5'-GTTTGATCCTGCATGCAAAAG- 3' and 5'-AGAGCAAACGATGTAGATCTCCTG –3. The PCR reaction (20-σl) contained 16 ng/σl template DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2-µM dNTPs, 0.2-µM each of the primers, 0.2U Taq polymerase. The amplification was carried out in a programmable thermal controller (MJ Research, Inc, MA) programmed as follows: 94°C for 2 min (94°C for 30 sec, 50°C for 45 sec, 72°C for 30 sec), for 35 cycles; 72°C 8 por min. The products were separated on a 1.2% agarose gel by electrophoresis, stained with ethidium bromide and photographed under UV light.

Activity 1.16. Identification of White Grubs Complex and its Natural Enemies in Antioquia

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Highlights:

- ∉ Key pests and their natural enemies in Antioquia identified
- ∉ Yield losses due to white grub attacks in Antioquia identified

Rationale

Soil pests cause serious economic losses to many important crops in South America. Until about 20 years ago white grubs were not considered important pests in Latin America. However, in the recent past outbreaks are no longer seasonally restricted as before and are present in many agro-ecosystems, such as

hillsides, tropical lowlands, including savannas and forest margins in Central and South America (Posada., 1993, Agricultura Tropical 30: 71-79; Londoño & Pérez, 1994, Rev. Colomb. Entomol. 20: 199-206; Shannon & Carballo, 1996, CATIE Informa Tecnico No. 227).

Inappropriate agricultural methods like inadequate cropping patterns, burning of harvest residues, deforestation, cultivation of marginal land, discontinuance of tillage, loss of floral and faunal biodiversity, reduction of organic material, ill-timed and excessive applications of synthetic pesticides and elimination of natural enemies of pests are considered to be the key factors responsible for the increased pest status of white grubs (Posada, 1993, Agricultura Tropical 30: 71-79; Londoño & Pérez, 1994, Rev. Colomb. Entomol. 20: 199-206). The frequent use of highly toxic soil pesticides can lead to development of resistance in pests and is additionally very often ineffective.

The populations of white grubs have increased in Eastern Antioquia since 1988 and are causing severe damage on a wide range of crops. In 2002 CIAT started collections in Northern and Eastern Antioquia. The quantity of individuals that we have collected is enormous: approximately 190,000 specimens until December 2003 (CIAT, PE-1 Annual Report 2004). In the present study we identified the most important white grubs that are considered as soil pests, described their feeding behavior, and estimated the damage that they caused on potatoes.

Materials and Methods

Description and definition of soil pests (diagnostic): We selected the sites of survey according to records of ICA and the Departmental Secretary of Agriculture. The Umata's helped us to identify potato and pasture farms where white grubs are traditionally a problem. The selected municipalities in Eastern Antioquia were characterized by similar physiographic characteristics and a mean altitude of 2100 m.a.s.l.: Rionegro, Marinilla, San Vicente, and El Carmen de Viboral and La Union (2600 m.a.s.l.). In Northern Antioquia we surveyed potato and pasture fields in San Pedro, Santa Rosa, and Entrerríos, all of them agroecologically similar zones at an altitude of approximately 2700 m.a.s.l. We surveyed the potato fields at harvest in order to avoid damage of the crop. In every field we evaluated 10 randomly selected square meters.

Determine yield losses due to white grub attacks: In order to determine the damage on potatoes and pasture due to attacks of white grubs and other soil pests we tabulated the information obtained from each surveyed square meter in the three agroecological zones of Northern and Eastern Antioquia and La Union. The parameters in potato fields were: number of plants, number o potatoes, number of potatoes damaged by white grubs, and number of potatoes damaged by other soil pests. In pasture we only tabulated the area (m^2) damaged white grubs.

Type of Damage: We set up experiments in the greenhouse for estimating damage on potato tubers in order to define the pest status of the white grub genera *Ancognatha*, *Cyclocephala*, *Ceraspis*, *Astaena*, and *Heterogomphus.* We collected white grubs during seven surveys in the savanna of Northern during August, September, October, and November in 2004 and took them to the greenhouse in the Research Station "La Selva" where the larvae fed on potato tubers. We also received 56 larvae (*Clavipalpus*, *Ancognatha*, *Astaena*, and *Cyclocephala*) from the departments Boyacá and Nariño. We revised the tubers once a week distinguishing the damage in four classes: normal (no damage), rasped, bitten, and burrowed (Figure 1.16.1). We identified the insects after having completed their development as adult.

Results and Discussion

Description and definition of soil pests (diagnostic): We continued the collection of white grubs and adults in Eastern and Northern Antioquia. In the cold climate of Northern Antioquia we collected 8.62±9.65 white grubs in one square meter. The white grub complex of this zone is detailed in Table 1.16.1. *Ancognatha* was by far the most dominant genus, followed by *Cyclocephala*.

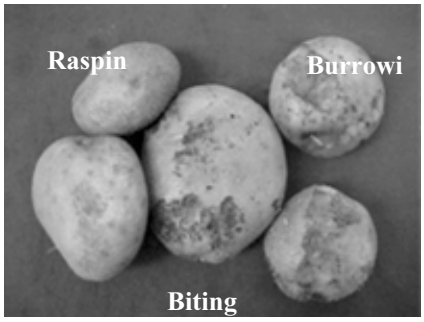

Figure 1.16.1. Types of damage caused by white grubs on potato tubers (Photo: CORPOICA)

Table 1.16.1. Number of collected white grubs from December 2002 until February 2004 in the cold climate of Northern Antioquia.

	Nº of collected specimens				
Municipality	**Entrerríos**	**San Pedro**	**Santa Rosa**		
Altitude ± SD m.a.s.l.	2384 ± 24.6 m.a.s.l.	2600 ± 7.9 m.a.s.l.	2584 ± 130.6 m.a.s.l.	**Total**	**%**
Ancognatha	43	83	275	401	73,6
Not identified	4	7	61	72	13,2
Cyclocephala	2	6	53	61	11,2
Astaena	0	0	7	7	1,3
Phyllophaga	0	1	2	3	0,6
Plectris	1	0	0	1	0,2
Total	50	97	398	545	100,0

In themoderate cold climate of Eastern Antioquia we collected 7.10±11.5 grubs per square meter. The proportion of genera presents differences between the surveyed sites. We collected 489 collected specimens of *Ancognatha* that was the most abundant genus in El Carmen de Viboral (Table 1.16.2). In the municipalities Rionegro and Guarne we found less grubs, however, the dominant genus were *Phyllophaga* and *Anomala*. We hypothesize that these marked differences are due to the elevated altitude of El Carmen favoring members of the subfamily Dynastinae such as *Ancognatha* spp. We observed that soil organic matter (SOM) is abundant in the colder zone and that is this subfamily prefers this food type. This observation was corroborated by the findings by Zuluaga *et al*. (2005, Feeding behavior of three white grub species associated with potato in the Savanna of Bogotá, manuscript in preparation) who report that *Ancognatha* spp. thrives best on SOM. It would be interesting to conduct soil analyses considering variables like SOM, pH, or altitude in order to understand the prevalence of this white grub group in determined areas. In the cold climate of La Union in Eastern Antioquia (2416 m.a.s.l.) we collected

8.5±12.38 grubs/m^2. Similar as in high altitudes of Northern Antioquia dominated the genus *Ancognatha* (Table 1.16.3). Surprisingly, we collected less white grubs in pasture than on potatoes. The number of specimen varied a lot. In the North we found 3.65±8.44 grubs / m^2, in the East 14.27±28.9 and in La Union 0.60±1.04.

Table 1.16.2. Number of collected white grubs from December 2002 until February 2004 in the moderate cold climate of Eastern Antioquia.

	Nº of collected specimens					
Municipality	**Rionegro**	**Guarne**	**El Carmen**	**San Vicente**		
Altitude ± SD m.a.s.l.	2175 ± 32.61 m.a.s.l.	2336 ± 76.04 m.a.s.l.	2260 ± 4.57 m.a.s.l.	2200 ± 0.00 m.a.s.l.	**Total**	**%**
Ancognatha	5	8	489	24	526	55,5
Not identified	41	18	0	132	191	20,1
Anomala	6	66	0	23	95	10,0
Phyllophaga	73	2	1	6	82	8,6
Cyclocephala	29	2	0	1	32	3,4
Plectris	0	2	5	6	13	1,4
Astaena	4	0	0	5	9	0,9
Total	158	98	495	197	948	100

Table 1.16.3. White grub complex in the cold climate of Eastern Antioquia (survey from December 2002 until September 2003

	Nº of collected specimens	
Municipality	**La Union**	**%**
Altitude ± SD m.a.s.l.	2416 ± 71.65 m.a.s.l.	
Ancognatha	530	96,9
Cyclocephala	17	3,1
Not identified	10	1,8
Total	557	100,0

Determine yield losses due to white grub attacks: We revised during 14 months 670 m^2, of those 72.8% harbored tubers that presented some type of damage. In Northern Antioquia we revised 12,257 tubers with an average of 49±19.17 tubers per square meter. We found on 10.15% of all revised tubers some damage that was caused by white grubs (7.51%), the potato tuber moth (*Tecia solanivora*) (1.58%), the Black Cutworm (*Agrotis ipsilon*) (0.08%), other insects (0.01%) and diseases (0.97%).

In Eastern Antioquia we revised 8509 tubers. Similarly to the north we found on 9.74% of the tubers symptoms of insect attacks. 5.89% of the damage was caused by white grubs, 3.35% by *T. solanivora*, 0.08% by *A. ipsilon*, 0.11 by other insects, and 0.32% by diseases. In the cold zone of La Union we checked 8289 tubers, of these 7.44% presented symptoms of damage: grubs (3.81%), *T. solanivora* (1.82%). Damage caused by other insects and diseases was about 0.2%.

Type of Damage: Larvae of the genus *Heterogomphus* never attacked potatoes indicating that they feed on decaying organic matter (Figure 1.16.2) confirming the observations by Zuluaga *et al.* (manuscript in preparation). A few *Plectris* larvae rasped the potato surface. *Ceraspis* and *Astaena* prefer to rasp the tuber; however, some larvae of these genera also bit the potato. *Ancognatha* and *Cyclocephala* can cause damage to root by biting, burrowing, and rasping. This corroborates the reports of Zuluaga *et al*. (manuscript in preparation) that *Ancognatha* can be a major pest problem when soil organic matter is not sufficiently available. The observation that *Cyclocephala* feeds on fresh plant material is new; however, it wouldn't be a surprise if these grubs may also act as pests when soil conditions force them to do so. We suggest conducting further experiments in order to understand the feeding behavior of this genus.

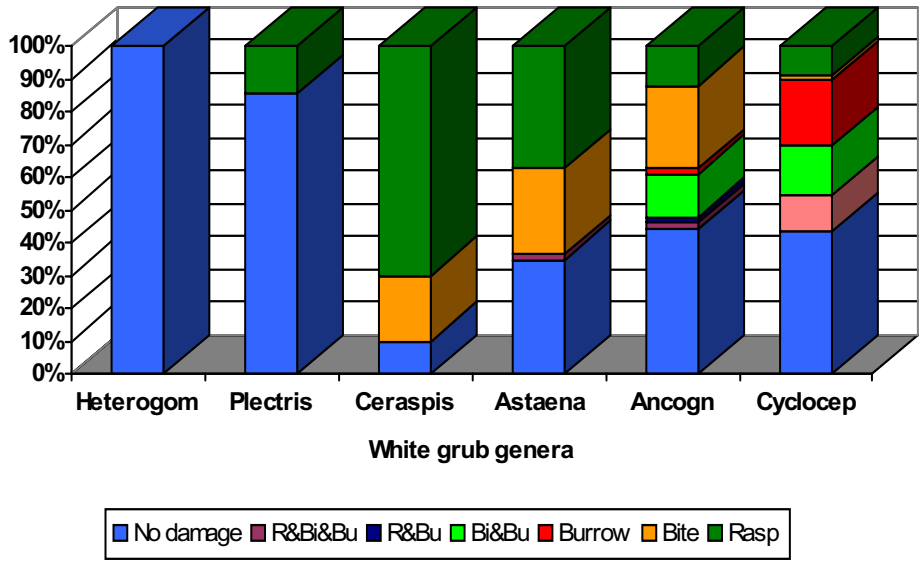

Figure 1.16.2. Feeding behavior of the white grub genera *Heterogomphus* (Heterogom), *Plectris*, *Ceraspis*, *Astaena*, *Ancognatha* (Ancogn), and *Cyclocephala* (Cyclocep). The larvae were collected in the field and the experiment was conducted in the greenhouse. Abbreviations in Legend: R= Rasp, Bi= Bite, Bu= Burrow.

Activity 1.17. Mass rearing *Phyllophaga menetriesi* (Col.: Melolonthidae) under controlled conditions

Contributors: G. A. Calberto, O. H. Yela Delgado, R. Zúñiga, and A. Gaigl

Highlight:

∉ Methodology for mass rearing of white grub species developed

Rationale

Numerous insects feed on the foliage or roots of bushy or tree species. Sometimes, the larvae of some are economically significant, as in the case of several species of the family Melolonthidae (or Scarabaeidae Pleurosticti), which can greatly affect the vegetation of their habitats. In tropical and subtropical America,

these white grubs, also known as *coro, joboto, chisa*, or *mojojoy*, number more than 500 species throughout the region. Yet, we are barely learning the basics of their taxonomy and ecology. Colombia presents a great diversity of beetles, some of which are of agricultural interest as adults are defoliators or leaf eaters, and their larvae are root-eating pests. Of the latter, larvae of the genus *Phyllophaga* stand out for their economic importance as they feed on the roots of various tropical and subtropical crops of the Americas, including cassava, maize, and vegetables, reducing their yields by either weakening or killing the plants.

Because this genus has a broad range of hosts, farmers mostly use chemicals to control these pests. However, precisely for being chemical, this type of control has proven inefficient in managing the pests and, above all, has had a severe impact on the environment. The rhizophagous larvae are protected by the soil, making control highly complex. Only by collecting basic information can technologies be developed to adequately and rationally manage the pests in their agroecosystems, thus increasing productivity at minimal economic and environmental cost, and permitting the preservation and use of biodiversity.

To seek the most viable strategy for combating the pests and generating suitable recommendations for their control, methodologies of mass rearing must be developed to obtain disease-free larvae that develop uniformly. Thus, they can be used for testing with entomopathogenic fungi, bacteria, and nematodes (Hidalgo *et al*., 1993, Rev Man Integ Plagas Hond 56:14–20)

The research discussed below was carried out at the International Center for Tropical Agriculture (CIAT). Its goal was to mass-rear *Phyllophaga menetriesi* Blanchard, a species belonging to the economically most important white-grub complex in southwestern Colombia. The methodology has been steadily improved and, currently, it can raise 8000 larvae per year.

Materials and Methods

Capturing adults: The site selected for capturing adults was in the Village District of Pescador, Department of Cauca, located at 1500 m above sea level (masl). In this site, 20-W, black-light traps were permanently installed in a cleared and visible place. These traps are in common use and are based on North American models of vertical light. They comprise basically three parts: a source of luminous radiation, a capturing device, and a collection container (Montoya *et al*., 1994, Rev Colomb Entomol 20:130–136). The traps were operated every night from 18:00 to 6:00 during October and November, when adults were most likely to be abundant and diverse (Pardo Locarno, 2002, MSc Thesis, Univ. del Valle, Cali, Colombia).

In Pescador, where nine traps had been set, the farmer's family members believed in the importance of the work, such that they daily surveyed the light traps. Their task was to separate the adults, initially by coloring and size (Ruiz and Posada, 1985, Rev Colomb Entomol 11: 21–26), and then place them in 5-L trays containing substrate that was previously sterilized at CIAT. Pieces of ripe plantain and carrot were used for food. At the same time, traps were set, one for each of three farms, in the Department of Quindío, where the insect's presence had been monitored in previous years (Pardo Locarno *et al*., 2003, *In*: Aragon et al., (eds), Estudios sobre coleopteros del suelo en America, Special publication, Univ. Autonoma de Puebla, Mexico, pp 45-63). The farmers made daily collections from these traps and the collected material was sent weekly to CIAT. As check, two black-light traps were installed in an area from which the chisa complex has not been reported: one trap was set in the Village District of El Olivo (1637 masl) and the other in the Village District of Potrerillo (1420 masl), both of the Municipality of Palmira (Valle del Cauca).

To facilitate different tests with entomopathogens, four light traps were installed at the Experiment Station "Tulio Ospina" of the Instituto Colombiano Agropecuario (ICA) in the Municipality of Bello (Antioquia),

10 km north of Medellín. The traps were set between January and April 2005, when the largest populations of adult Melolonthidae appear in this area (Montoya *et al*., 1994, Rev Colomb Entomol 20:130–136). The two strains were combined into a colony to ensure two periods of flight although, in the field, this species is univoltine only.

Although in eastern Antioquia, *Phyllophaga obsoleta* Blanchard stands out for its economic impact (Pardo Locarno *et al*., 2003, *In*: Aragon et al., (eds), Estudios sobre coleopteros del suelo en America, Special publication, Univ. Autonoma de Puebla, Mexico, pp 45-63; Vallejo *et al*., 1998, Coleopterist's Bull 52:109–117), *P. menetriesi* has also been reported as being present. Thus, traps were set to capture adults and so determine the abundance of this species in the area. Captures of *P. menetriesi* carried out previously at the station increased the number of adults collected for mass rearing. The adults from this area were collected daily and placed in 10-L plastic trays. The number of captured adults was recorded. Because of the efficiency with which this task was carried out, we could obtain two breeding periods at CIAT: October–November and April–May.

The mass-rearing methodology was carried out at the Quindío Laboratory of CIAT's Cassava Entomology Project, located in the Municipality of Palmira (Valle del Cauca; 965 masl). At the same site, a trap was also set to capture adults. However, results were very poor.

Conditions for mass rearing were an average temperature of 23 ± 2 ºC and a relative humidity of 80% ± 10%. The substrate consisted of soil from the organic or humus layer mixed with topsoil in a 1:1 ratio. The mixture was passed through a mesh with a 5-mm pore size (Hidalgo *et al*., 1993, Rev Man Integ Plagas Hond 56:14–20). It was then steamed for 4 h in a greenhouse soil sterilizer. The sterilized soil was left to stand for 48 h or more to eliminate the toxic gases that accumulate during the soil's heating and which could kill the insects (Hidalgo *et al*., 1993, Rev Man Integ Plagas Hond 56:14–20).

Oviposition: An adult *P. menetriesi* measures 18 to 23 mm, and is dark brown. Its body is robust, being broader towards the rear than at the head. The entire body surface is covered with scorings and dense, brown, and regularly distributed bristles or setas. It is characterized by a sinuate pronotal margin, an erect and somewhat raised clypeus, and metatibial articulated spurs (Figure 1.17.1).

Figure 1.17.1. Typical adults of *Phyllophaga menetriesi*, an economically significant crop pest in Colombia. (A) male, (B) female

The captured adults were separated by sex, according to the external characteristics of their genital orifice (Hidalgo *et al*., 1993, Rev Man Integ Plagas Hond 56:14–20), which presents diphormism in the last abdominal segment (Figure 1.17.2). Fifteen couples of adults were placed in 10-L plastic containers, which had covers made of coarse canvas with metal edges to prevent individuals escaping while

permitting air to circulate within the containers. The containers contain about 7 L of sterilized soil, which was watered every 3 days to prevent the soil from drying. Leaves of *Erythrina* spp. (Fabacae) and West Indian cedar or *guácimo* (*Guazuma ulmifolia* Lam.) were also added to feed the adults (Figure 1.17.3). The plant material was constantly replaced and dead adults removed every 8 days to prevent the accumulation of decomposing insects. Genitalia were also extracted from dead males, and mounted on entomological pins for later verification of identification. If the adults were alive, they were returned to the container and the soil watered again.

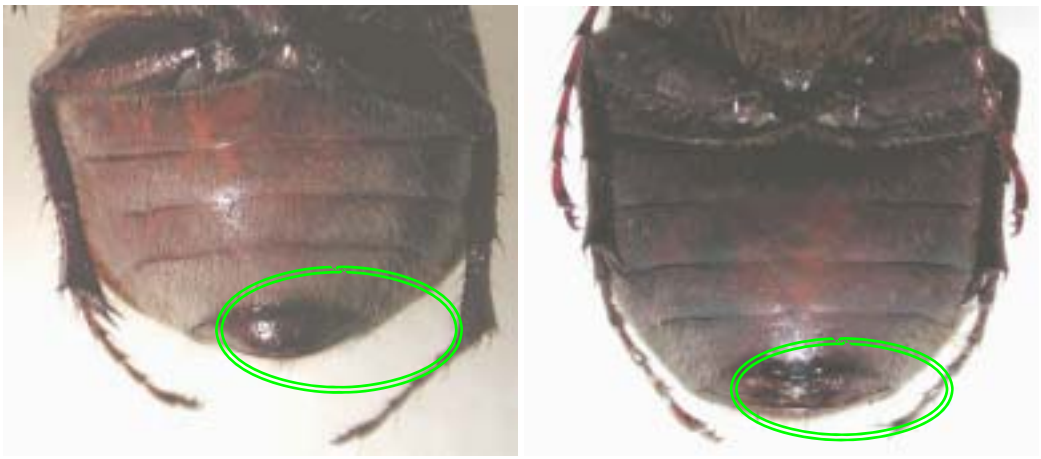

Figure 1.17.2. Diphormism of the last abdominal segment in adult *Phyllophaga menetriesi*. On the left is a male, with a female on the right.

Figure 1.17.3. Ten-liter plastic containers for carrying soil and plant materials for mass-rearing *Phyllophaga menetriesi*. Fifteen couples of adult beetles are placed in each container.

Extracting eggs: Eggs were extracted every 8 days. All the soil was removed from the plastic containers and any eggs removed manually with a spatula, placing them into plastic trays (Hidalgo *et al*., 1993, Rev Man Integ Plagas Hond 56:14–20). They were then separated, 25 eggs per 16-oz plastic container, each of which was duly labeled with the date of placement (Figure 1.17.4). Carrots were also added as food for the

first-instar larvae (Figure 1.17.5), thus preventing cannibalism, which appears from first-instar stage onwards.

The eggs were examined every 8 days to check which ones were close to hatching. Eggs are at first white and round. As they incubate they increase in size, averaging 2.7 mm in length (Ruiz and Posada, 1985, Rev Colomb Entomol 11:21–26). *Phyllophaga menetriesi* females oviposit their eggs individually in a chamber shaped like a clod of earth, according to Ruiz and Posada (1985, Rev Colomb Entomol 11:21–26) when describing *Ancognatha scarabaeoides* Erichson. Each egg appears covered with particles of the substrate used (Figure 1.17.6a). When the egg is fully developed, the jaws that break the chorion can be distinguished.

Figure 1.17.4. Sixteen-ounce plastic containers carrying soil with eggs of *P. menetriesi*.

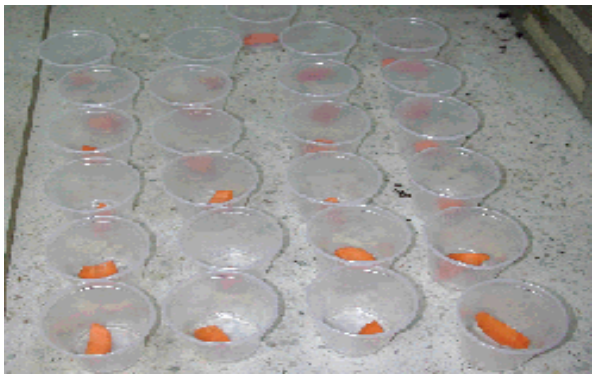

Figure 1.17. 5. Carrot pieces for feeding larvae of *P. menetriesi* are placed in the bottom of 16-oz plastic containers before soil and the pest's eggs are added.

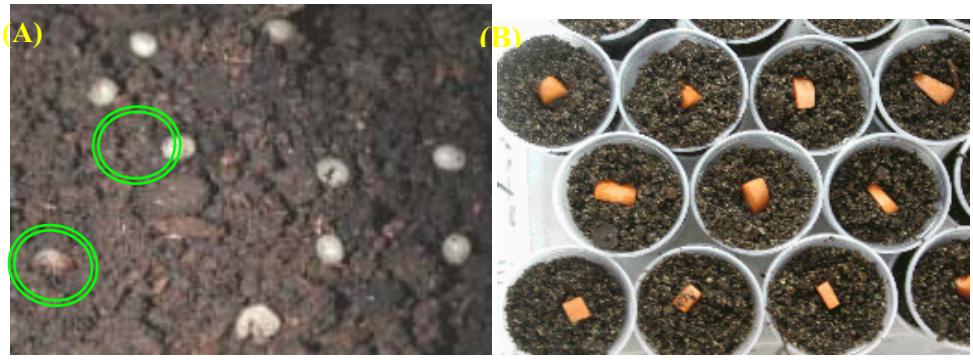

Figure 1.17. 6. (A) Eggs (top circle) and first-instar larvae (bottom circle) of *Phyllophaga menetriesi*, a crop pest. (B) First instars are removed and placed into 4-oz plastic containers carrying soil and carrot pieces

Eclosion and development of the first instar: After eclosion, the larvae were individually placed into 4-oz plastic containers with covers. The soil had the same characteristics as used for oviposition. Carrot pieces were added to feed the larvae (Figure 1.17.6b) and replaced every 15 days, when they were estimated to be totally consumed.

At this stage, care is essential for managing individuals as they are susceptible to any adverse condition occurring during their development. Critical conditions include the presence of dense root masses, loss of soil moisture, and excessive manipulation. A mortality rate of 45%–50% is considered normal for this stage, although the rate for the next stage may drop to 15%.

Second instar: Eighteen to 30 days after eclosion, about 85% of the larvae pass to second instar, developing in the same plastic containers (Figure 1.17.7a). Once they reached the second instar, the soil and food were replaced. Instead of carrots, rice seedlings, which have a larger number of roots that can cater for the larvae's increased consumption, were used. Hence, the larvae could develop without anomalies. To determine if the larvae were in second instar, the cephalic capsule was examined; it should have shown considerable increase, of about 38% (Calberto, 2004, Thesis on Environmental Administration, Faculty of Basic Sciences, Universidad Autónoma de Occidente, Santiago de Cali, Colombia, 65 p) (Figure 1.17.7b).

Third instar: Once they reached third instar (Figure 1.17.8), the larvae, now measuring 34 mm long and with a 5.2-mm-wide thorax, were put in 10-oz plastic containers. The food was changed back to carrot, this time in 5 to 7-g pieces (Calberto, 2004, Thesis on Environmental Administration. Faculty of Basic Sciences, Universidad Autónoma de Occidente, Santiago de Cali, Colombia, 65 p). The change in diet made it possible to reduce observations. The food was changed once a month, as this is the most voracious instar (King, 1984, Trop Pest Man 30: 36–50). The units had to be continually checked to maintain adequate soil moisture and keep the larvae in good condition. During the change from this instar to prepupa, care had to be taken with food spoilage, hastened by the larvae dumping their stomachical contents on the carrot. This dumping is necessary for the pupal chamber's formation because it acts as a compactant, making the chamber solid and giving it an environment that is suitable for the insect's development. At the end of the third instar, the larvae suspended feeding, not requiring plant materials to complete their later stages of prepupa and pupa.

Pupa and imago stages: The pupae stayed in the same containers and under the same conditions in which they developed as larvae. Every 15 days, we checked their progress and looked for precocious adults. Great care was taken to ensure no harm came to the insects while handling them during this delicate stage

of metamorphosing into the adult state. Equally essential was to prevent any damage to the earthen pupal chamber itself. Once damaged, it could not be repaired and would interrupt the insect's development for a considerable time (Figure 1.17.9).

The adults were then placed in 10-L plastic containers carrying sterilized soil and left for 70 to 75 days in their pupal chambers so that their reproductive organs could develop. During this time, they were not given moisture or food. Some adults were mounted on entomological pins to confirm their taxonomic identification and to verify the progeny of parents and children.

After mass rearing, a group of 300 eggs were selected to examine the life cycle of *P. menetriesi* under controlled conditions. Previous experiences with mass rearing led to the use of 400 individuals to compare differences of mortality rates resulting from the use of rice or carrot to feed first-instar larvae. Another important aspect that was compared was differences in percentage of eclosion of eggs oviposited by field females versus those that were mass reared.

Figure 1.17.7. (A) Two technicians individualize first and second-instar larvae of *P. menetriesi*. (B) First (1) and second (2) instars of *P. menetriesi.*

Figure 1.17.8. Third-instar larvae of *P. menetriesi*. (A, left) Early third instar. (B) Prepupal stage.

Figure 1.17.9. From pupa to imago: (A) a third-instar larva had constructed its pupal chamber at the bottom of a 10-oz plastic container (the contents are shown inverted on to the work bench). Pupation takes about 5 weeks. (B) and (C) The fully developed insect ruptures its chamber, pushing the two halves apart, and begins scrambling out. (D) Imago rests beside its pupal molt. It remains underground for about 10 weeks before emerging above ground.

Results and Discussion

Adults captured by region: We collected a total of 9439 adults between October and November 2004, and March and April 2005. Figure 1.17.10 shows the geographic distribution of the captures. Because the largest number of captures was in Cauca, we opted to increase the number of light traps in this Department to more exhaustively collect adults and thus increase the existing breeding stock in the mass-rearing installations.

Monitoring the life cycle of Phyllophaga menetriesi: The study of the insect's life cycle began with 300 eggs, from which we obtained 226 first-instar larvae > 192 second instars > 179 third instars > 160 pupae > 138 adults. The eggs incubated for 10 to 16 days, with an average of 13 days. The first instars developed over 19 days; the second instars took 15 to 32 days, with an average of 27 days, and the third instars took 82 to 201 days, with an average of 175 days. (Table 1.17.1).

As reported by King, (King, 1996, Technical report No. 277 CATIE, p 50-61), we found that, after the third instar, the insects entered a stage of diapause that lasted about 30 days before they became pupae. The pupal stage averaged 34 days. The adults remained in their pupal chamber for about 73 days. Their flight period lasted 15 days. The adult stage therefore lasted 88 days. The entire development of *P. menetriesi* from egg to adult averaged 386 days (Figure 1.17.11).

Table 1.17.1. Life table of the crop pest *Phyllophaga menetriesi*, under mass-rearing conditions.

X	L_x	d_x	q_x	E_x	T_x	e_x
Egg	300	74	0.247	263.0	1045.0	3.97
First instar	226	34	0.150	209.0	782.0	3.74
Second instar	192	13	0.070	185.5	573.0	3.09
Third instar	179	19	0.106	169.5	387.5	2.29
Pupa	160	22	0.137	149.0	218.0	1.46
Adult	138	138	1.000	69.0	69.0	1.00

Where,

X is Age interval in units of time
L_x is Number of live individuals at the beginning of the interval x to x + 1
d_x is Number of individuals that died during the interval x to x + 1
q_x is Mortality rate during the interval x to x + 1
E_x is Average number of live individuals during the interval x to x + 1
T_x is Cumulative sum of L_x to obtain values expressed in number of individuals per unit of time
e_x is Average life expectancy of individuals at the beginning of interval x

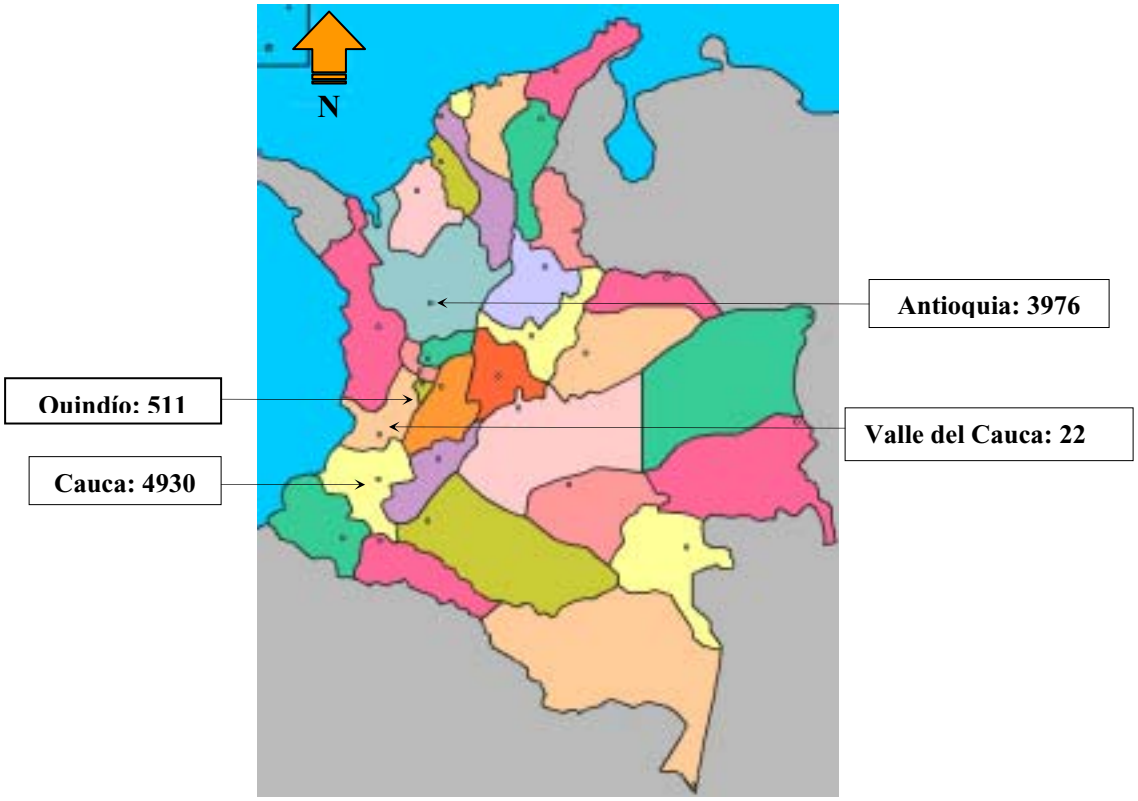

Figure 1.17.10. Colombia and its departments. Numbers indicate the quantity of adult *Phyllophaga menetriesi* captured per department.

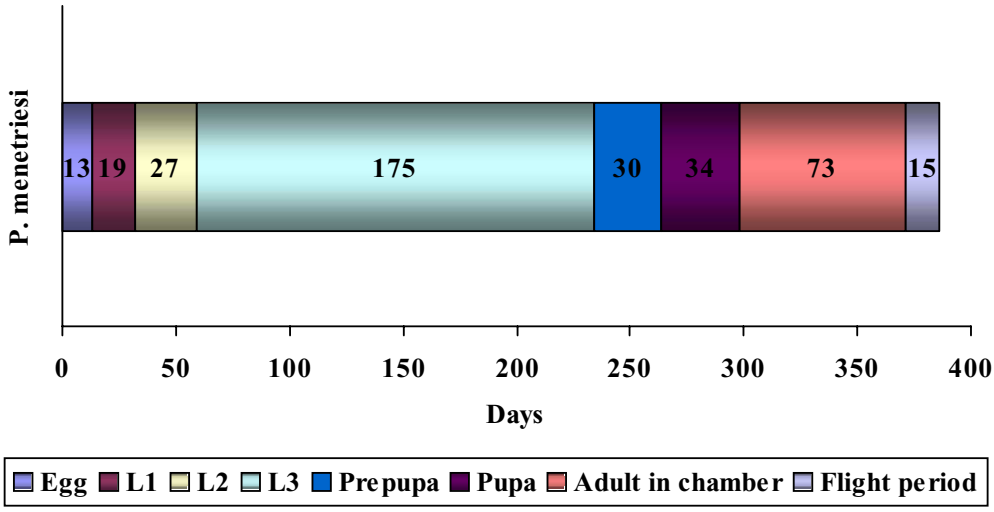

Figure 1.17.11. Average duration of the developmental stages of *Phyllophaga menetriesi*. The total life cycle lasts about 386 days. L1 = first-instar larva; L2 = second-instar larva; L3 = third-instar larva.

Comparing eggs from field females with those from mass-reared females: For this experiment, mass-reared and field individuals were used. Following the proposed methodology for mass rearing, the eggs were separated by origin and placed in 16-oz pots, each with 25 eggs. In all, we compared 2100 eggs distributed in 84 pots.

The percentage of eclosion of eggs obtained from laboratory females was 34.68%, whereas that of field females was 75.30% (Table 1.17.2). We also observed that a laboratory female would oviposit, on the average, 9 eggs per week, with two oviposition events at an interval of 7 days, meaning that she would oviposit 18 eggs during her adult life. In contrast, the field females oviposited, on the average, 2 eggs during a single oviposition event within 7 days.

Table 1.17.2. Oviposition data for *Phyllophaga menetriesi*. Adult females from the field were compared with those reared in the laboratory.

Origin	Eggs (no.)	Days of oviposition	Eclosion (%)
Mass-reared	9	14	34.68
Field	2	7	75.30

Mortality rates of first-instar larvae according to food type: To optimize mass rearing, a sample of 400 first-instar larvae were compared according to the different foods they were fed. Half were given pre-germinated rice and the other half, carrot pieces. The mortality rate from first to second instar was 15.5% with carrot—close to the 15% obtained with the mass-rearing methodology—and with rice, the rate was 45% (Table 1.17.3).

In the containers carrying rice, the roots had proliferated, which led to soil compaction and the consequent drowning of the larvae. In the containers carrying carrot, soil moisture increased only slightly and a fungus also appeared on the food, although it was not detrimental to the larvae's development. Results

therefore suggested that work should continue with carrot as it does not interfere significantly with the larvae's development and is easier to handle.

Table 1.17.3. Mortality rates of first instars of the crop pest *Phyllophaga menetriesi* according to the type of food they received.

Food	Individuals (starting number)	Dead individuals (no.)	Mortality (%)
Carrot	200	31	15.5
Rice	200	90	45

Observations

The laboratory larvae were smaller than the field samples because of being confined throughout their development in the laboratory. The adults were also smaller than their field counterparts. The capacity of the mass-reared insects to oviposit needs to be studied.

The maintenance of adults and larvae in containers with perforated covers that permit the exchange and circulation of air must be considered when selecting materials for use during mass-rearing procedures.

For oviposition, the soil must be rich in organic matter, as preferred by females, so to give them a suitable medium for carrying out this task. At the beginning of each instar, the larvae possess a white head, with the exuviae attached to the lower parts of the body. The exuviae quickly degrade in the soil to the point of being completely removed. Sometimes, however, part of the cephalic capsule can be found during molting.

Care must be taken during first instar with the amount of food placed in the containers because excess food may produce a large number of rootlets that compact the soil, leading to the larvae drowning. Nevertheless, soil moisture must be kept constant throughout the life cycle as all stages are susceptible to unfavorable substrate conditions. Care also needs to be taken with excess moisture, which will weaken the larvae, causing death, and in adults cause inadequate extension of the elytra and sagging of the abdomen.

Larvae of *P. menetriesi* constantly feed until they reach the point of becoming prepupae when, just before becoming immobilized, they expel, in one event, their stomachical contents, thus humidifying the substrate and decomposing the food, which becomes gelatinous. *Phyllophaga obsoleta* and other species studied in Mexico also demonstrate this behavior.

Activity 1.18. Evaluating the Impact of Biotechnology on Biodiversity: Effect of Transgenic Maize on Non-Target Soil Organisms

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Highlights:

- Standardized protocols developed for risk evaluations of GMOs on non-target soil organisms.
- No statistical differences were detected in abundance and diversity of soil organisms in conventional versus genetically modified cotton [Bollgard ® Bt Cry 1A ©] during the 2003-05 period in the Cauca Valley, Colombia.

- Evaluations of the Arthropod family Formicidae and the order Coleoptera during 2004-05 found no significant differences on conventional versus non-conventional cotton (Bt) in the Cauca Valley, Colombia.

- Basic knowledge on biological aspects of the class Collembola, an important soil Arthropod was enhanced. Information on Collembola distribution associated with cotton, maize and pastures in five Colombian Department is available. A references collection for future taxonomic studies has been developed and duplicate collections have been established at the Universidad Nacional en Bogotá and at Cornell University.

Rationale

Despite the controversy over the use of genetically-modified organisms (GMOs), the number of countries with GM commercial crops has grown from one in 1992 to 13 in 1999 (Shelton *et al.,* 2002, Ann. Rev. Entomol. 47: 845-81.). From 1996 to 2000, the global area under GMOs increased 42.5 million ha (James, 2004, www.ISAA.org, cited in May 27, 2004). The countries that reported the greatest increases were USA, Canada and Argentina, with 98% of the total area (Shelton *et al.*, 2002, Ann. Rev. Entomol. 47: 845-81). During 2003/04 67.7 million ha of GMOs were planted worldwide, of which soybean, maize, cotton and colza occupied 99% of the total area. Soybean plantings made up 61%, followed by maize and cotton with 23 and 11%, respectively (ICAC Recorder 2004, www.**icac**.org/**icac**/cotton_info/ cited in July 10, 2004).

For 2003/04, the area planted to GM cotton was 6.8 million ha, representing 21% of the total area planted to cotton worldwide. The countries that currently plant GM cotton are Argentina, Australia, China, Colombia, India, Indonesia, Mexico, South Africa and USA. The proportion of cotton that was GMO in 2003/04 was 77% for USA and 58% for China with an increase of 7% with respect to the previous harvest (ICAC Recorder 2004).

The Ministry of Agriculture and Rural Development, through the Colombian Agricultural Institute (ICA), designed a scheme to determine the viability of incorporating GMOs into the agricultural production system. ICA therefore passed Resolution 03492 in 1998 to establish and regulate the process of introduction, production, liberation and commercialization of GMOs. In two other provisions (Agreements 013/98 and 0002/02) ICA created the National Technical Council of Agricultural Biosecurity (CTN) to function in the assessment and support of GMO technology.

Since the establishment of those regulations, applications have been submitted for *Brachiaria*, carnations, cassava, coffee, cotton (resistance to lepidopterans), maize, rice, *Stylosanthes* and sugar cane. Of these, only four have been approved to date: (i) carnations for cut-flower production, (ii) cotton for commercial production, (iii) rice for small scale field trials, and (iv) maize for biosecurity tests (Díaz, 2003, Informe al Consejo Técnico Nacional de Bioseguridad (CTN), pp 8).

For the period 1991-2002, Colombia experienced a reduction of 83% in the area planted to cotton. The 2001/02 harvest only included 39,000 ha in the two cotton-growing regions of Tolima-Valle and Costa-Meta. One aspect that has greatly influenced the loss of area planted to cotton in Colombia is the high incidence of pests. The greatest losses are caused by the boll weevil (*Anthonomus grandis*, Coleoptera: Curculionidae) that affects 89% of the growing area in the departments of Córdoba, Cesar and Tolima, causing 15% loss of flower heads. *Heliothis virescens* (Lepidoptera: Noctuidae) affects 100% of the cotton planting area of Colombia, causing damage to 15-20% of the flower heads and bolls. Some 10% of the cultivated area is additionally affected by the foliovore "gusano rosado" (*Sacadodes pyralis*, Lepidoptera: Noctuidae) and whiteflies (Homoptera: Aleyrodidae).

Control of these pests is largely based on extensive use of agrochemicals and these represent 23% of the direct costs of the crop to the Colombian producer. In the Atlantic Coast, there was an average of 26 applications of pesticides per crop cycle, with 69.2% of those directed toward the control of lepidopterans. In the Cauca Valley, the number of applications has been reduced 73% to an average of 7 applications per crop cycle, with 57.1% directed towards the control of lepidopterans.

Given this scenario, ICA and the CTN implemented the first studies to determine the effect of the Bollgard® technology (Monsanto) on populations of arthropods and annelids in the cotton zone of Córdoba department in the Caribbean Region. The Bollgard® technology, generated by Monsanto, has the Cry1Ac insert whose target pests include the following lepidopterans: *Alabama argillacea* (Noctuidae, cotton leafworm), *Heliothis virescens* (Noctuidae, tobacco budworm), *Helicoverpa zea* (Noctuidae, corn earworm), *Pectinophora gossypiella* (Gelechiidae, pink bollworm), *Sacadodes pyralis* (Noctuidae, "el gusano rosado colombiano"), *Spodoptera frugiperda* (Noctuidae, fall armyworm), *Trichoplusia* sp. (Noctuidae, looper) and *Bucullatrix* sp. (Lyonetiidae, cotton leaf perforator)

Based on results obtained during the 2001-2002 growing cycle, ICA authorized the first commercial plantings of cotton with resistance to lepidopterans. The department of Córdoba was the first to commercially plant GM cotton with 6,187 ha planted in the second semester of 2003. During the first semester of 2004, 4,495 ha were planted in Tolima-Huila and 696 ha in the Cauca Valley.

Activity 1.18.1. Effect of transgenic cotton (Bollgard® Cry1A(c)) on Non-Target Soil Arthropods in the Cauca Valley of Colombia

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Materials and Methods

In collaboration with ICA's division of Agricultural Regulation and Protection, we initiated field studies for the first cycle of cotton at the ICA research station in Palmira, located at 03°31'N, 76°19'W, 975 m elevation, annual precipitation 1295 mm, mean temperature 24°C, relative humidity 76%, and corresponding to the Holdridge life zone of Dry Tropical Forest.

The evaluations were conducted within the methodology implemented by ICA to evaluate the effect of Bollgard® technology on arthropod populations in the cotton crop in the departments of Tolima, Huila and Valle del Cauca.

The experimental units were plots measuring 225 m^2 (15 m x 15 m) in a completely randomized block design. Each block had 6 plots for a total of 24 plots under evaluation. Plant material was (1) Bollgard® technology represented by the variety NuCont 33B that contains the Cry1A(c), and (2) the conventional technology represented by variety DP 5415 (Figure 1.18.1.1).

Figure 1.18.1.1. The experimental units in the Cauca Valley, Colombia

Sampling: Information was gathered from two types of samples: pitfall traps and berlese funnels. Pitfall traps were located between plants within the rows; eight were put out in each experimental plot (Figure 1.18.1.2). A total of 192 pitfall traps were deployed, and these were opened to sampling for a 24-hour period each week.

Figure 1.18.1.2. (A) Fixed component and removable component and (B) lid of the pitfall traps in the field.

Field samples were brought to the laboratory for their processing on the same day. Larger arthropods were picked out by hand. To recover the microarthropods, the samples were processed in a small funnel lined with a very fine mesh. The field sample was washed into the funnel with water. By capping the end of the funnel, the sample was floated, and the supernatant removed after discarding the larger debris. Then the remaining precipitate was floated again, this time in 35% salt solution and the supernatant removed. Both supernatant samples were then combined and stored in 70% ethyl alcohol until analysis and identification (Figure 1.18.1.3).

Figure 1.18.1.3. Cleaning and storage of samples in the laboratory.

In addition to the pitfall traps, a cup cutter was used to take soil samples every 2 weeks. The cup cutter had a diameter of 10 cm and the sample was taken to a depth of 10 cm in the row between plants (Figure 1.18.1.4). Four samples were taken per plot for a total of 96 samples per evaluation. Samples were placed in berlese funnels for 24 hours after which the samples of separated arthropods were stored in 70% ethyl alcohol until analysis. Because only 48 funnels were available, blocks 1 and 2 were done the first period, followed by blocks 3 and 4 which were maintained at 11^{o}C during the interim 24 hours. Arthropod samples were separated, sorted and processed as in the pitfall trap samples.

Figure 1.18.1.4. Field collection of samples for berlese extraction of arthropods using a "Lever Action Hole Cutter."

Analysis of information: The statistical model used for the analysis of the data was a completely randomized block design. With this design an ANOVA will be used to determine differences in abundance among treatments and determine the effect of their interactions. In addition, for the most abundant groups we will conduct an analysis of the area under the population curve (accumulated insect-

days) to determine differences among treatments during the trial. We will also compare the diversity and abundance among treatments using various indices of taxonomic diversity, dominance and equity.

Results and Discussion

Arthropod Taxonomic Composition: During both cycles of evaluation (2003-2004), 1,167,928 specimens were captured representing 22 orders and 10 taxonomic classes (Tables 1.18.1.1, 1.18.1.2). The most abundant class was Aracnida with 49.8% of total individuals captured (Table 1.18.1.1). Of all individuals captured, 51.9% of those were associated with transgenic cotton and 48.1% with conventional cotton (Table 1.18.1.2, Figure 1.18.1.5). Of the 22 identified orders, the most abundant were Acari and Poduromorpha with 50.3 and 26.7%, respectively (Table 1.18.1.2). Only Chilopoda, Diptera, Neuroptera, Poduromorpha, Symphyla and Symphypleona exhibited a significant difference in abundance between treatments, with Poduromorpha and Symphypleona more abundant in modified cotton. Of taxa identified in the two cycles, only Araneae, Neuroptera, Orthoptera, Strepsiptera and Thysanura did not exhibit a significant difference in abundance between cycles (Table 1.18.1.3).

Table 1.18.1.1. Number of individuals and composition of arthropod classes caught in Pitfall traps and Berlese funnels in cotton, during 2003 and 2004 in the Cauca Valley, Colombia.

Class	DP5415	%	Nucotn	%	Total	%
Aracnida	295,793	52.7	286,318	47.2	582,111	49.8
Chilopoda	792	0.1	686	0.1	1,478	0.1
Collembola	162,459	28.9	223,465	36.8	385,924	33.0
Diplopoda	382	0.1	422	0.1	804	0.1
Diplura	907	0.2	821	0.1	1,728	0.1
Insecta	95,785	17.1	93,659	15.4	189,444	16.2
Malocostraca	213	0.0	247	0.0	460	0.0
Pauropoda	429	0.1	389	0.1	818	0.1
Protura	14	0.0	12	0.0	26	0.0
Symphyla	4,588	0.8	547	0.1	5,135	0.4
Sum	561,362	**100**	**606,566**	**100**	**1,167,928**	**100**

Pitfall traps: During both cycles (2003-2004), 574,814 individuals were captured, belonging to 19 different taxonomic orders and 3 taxonomic classes (Table 1.18.1.4). Of all individuals captured, 55.7% of those were associated with transgenic cotton and 44.3% with conventional cotton. Of the 19 identified orders, the most abundant were Poduromorpha, Hymenoptera and Acari with 51.2, 23.6 and 21.5%, respectively (Table 1.18.1.4). Abundance, in terms of individuals per order, was 1.3 times greater in NuCotn 33B; of all orders, only 9 were more abundant in DP5415 (Table 1.18.1.4). Only the orders Chilopoda, Isopoda, Neuroptera, Poduromorpha, Psocoptera and Symphypleona exhibited a significant difference in abundance between treatments, were more abundant in NuCotn 33B, excepting to Psocoptera (Table 1.18.1.4).

Given their overall abundance, the class Collembola and the order Hymenoptera were examined in more taxonomic detail. For the class Collembola, seven families were identified belonging to three orders (Table 1.18.1.5). Over both cycles of evaluation (2003 and 2004), Poduromorpha and Simphypleona exhibited statistical differences among treatments, being more abundant in modified cotton (NuCotn 33B) (Tables 1.18.1.3, 1.18.1.4). The order Hymenoptera represented 23.6% of total individuals captured, with 99.7% representing the family Formicidae where 52.1% were captured in DP5415 (Table 1.18.1.4).

Table 1.18.1.2. Number of individuals and composition of arthropod taxa caught in Bt cotton (NuCotn 33B) and conventional cotton (DP 5415), during 2003 and 2004 in the Cauca Valley, Colombia.

Orders	DP 5415	%	NuCotn 33B	%	Total	%
Acari	295,252	52.6	285,708	47.1	580,960	49.7
Aranae	541	0.1	610	0.1	1,151	0.1
Blattaria	57	0.0	43	0.0	100	0.0
Chilopoda[1]	792	0.1	686	0.1	1,478	0.1
Coleoptera	2,337	0.4	2,124	0.4	4,461	0.4
Dermaptera	26	0.0	30	0.0	56	0.0
Diplopoda[1]	382	0.1	422	0.1	804	0.1
Diplura[1]	907	0.2	821	0.1	1,728	0.1
Diptera	1,091	0.2	881	0.1	1,972	0.2
Entomobryomorpha	40,849	7.3	35,590	5.9	76,439	6.5
Hemiptera	144	0.0	119	0.0	263	0.0
Homoptera	4,165	0.7	3,864	0.6	8,029	0.7
Hymenoptera	87,249	15.5	82,754	13.6	170,003	14.6
Isopoda	213	0.0	247	0.0	460	0.0
Lepidoptera	260	0.0	266	0.0	526	0.0
Mantodea	3	0.0	3	0.0	6	0.0
Neelipleona	238	0.0	173	0.0	411	0.0
Neuroptera	9	0.0	1	0.0	10	0.0
Orthoptera	69	0.0	57	0.0	126	0.0
Pauropoda[1]	429	0.1	389	0.1	818	0.1
Poduromorpha	121,254	21.6	187,155	30.9	308,409	26.4
Protura[1]	14	0.0	12	0.0	26	0.0
Psocoptera	82	0.0	57	0.0	139	0.0
Strepsiptera	1	0.0	0	0.0	1	0.0
Symphyla[1]	4,588	0.8	3,701	0.6	8,289	0.7
Symphypleona	118	0.0	547	0.1	665	0.1
Thysanoptera	134	0.0	142	0.0	276	0.0
Thysanura	1	0.0	1	0.0	2	0.0
Unidentified	157	0.0	163	0.0	320	0.0
Sum	**561,362**	**100**	**606,566**	**100**	**1,167,928**	**100**

[1]Taxonomic Class (Including for analysis)

In the analysis of the area under the curve for Poduromorpha, Hymenoptera and Acarina, they presented the same behavior during the two evaluation cycles (Figures 1.18.1.5, 1.18.1.6, 1.18.1.7). Only Poduromorpha presented a change during the 2004, where more area under the curve was accumulated in NuCotn 33B.

Berlese funnels: Over both cycles of evaluation (2003-2004), 593,114 individuals were captured, representing 22 orders and 8 taxonomic classes of arthropods (Table 1.18.1.4). The 51.7% of individuals captured were associated with conventional cotton and 48.3% with transgenic cotton. The most abundant order was Acarina, with 77.2% of total captures and 1.1 times more abundant in DP 5415. Only the orders Acarina, Chilopoda, Coleoptera, Diptera, Entomobryomorpha and Symphyla exhibited a significant difference in abundance between treatments, were more abundant in DP5415 (Table 1.18.1.4).

Table 1.18.1.3. Abundance of arthropods (mean ± S.E. number of individuals caught per evaluation date) associated with cotton, during 2003 and 2004 in the Cauca Valley, Colombia.

Taxa	Between treatments		Between samples		Between cycles	
	DP 5415	NuCotn	Pitfall	Berlese	2003	2004
Acarina	50.4±149.9a	67.9±162.9a	18.4±52.7b	264.8±280.3a	54.1±104.4b	82.7±210.4a
Aranae	0.1±0.7a	0.1±2.4a	0.2±2.0a	0.1±0.3b	0.1±0.3a	0.2±2.5a
Blattaria	0.0±0.1a	0.0±0.1a	0.0±0.1a	0.0±0.2a	0.0±0.2a	0.0±0.lb
Chilopoda[1]	0.1±0.7a	0.2±0.8b	0.0±0.1b	0.8±1.6a	0.1±0.4b	0.3±1.0a
Coleoptera	0.4±1.5a	0.5±1.7a	0.2±0.5b	1.9±3.3a	0.7±2.1a	0.4±1.2b
Dermaptera	0.0±0.1a	0.0±0.1a	0.0±0.1a	0.0±0.1a	0.0±0.0b	0.0±0.1a
Diplopoda[1]	0.1±0.4a	0.1±0.5a	0.0±0.2b	0.4±1.0a	0.1±0.4b	0.1±0.6a
Diplura[1]	0.2±0.7a	0.2±0.9a	0.0±0.0b	1.0±1.7a	0.1±0.5b	0.3±1.1a
Diptera	0.2±1.0a	0.2±1.6b	0.0±0.6b	0.7±1.6a	0.3±1.3a	0.1±1.5b
Entomobryomorpha	7.0±27.7a	8.4±26.7a	1.6±6.8b	38.0±55.3a	11.0±34.1a	7.2±24.5b
Hemiptera	0.0±0.2a	0.0±0.2a	0.0±0.2a	0.0±0.2a	0.0±0.3a	0.0±0.1b
Homoptera	0.7±3.4a	0.9±2.8a	0.9±3.0a	1.2±4.6a	0.9±4.0b	1.0±2.7a
Hymenoptera	14.9±55.5a	19.6±64.0a	20.2±60.1a	20.0±78.0b	39.2±86.6a	1.9±14.7b
Isopoda	0.0±0.4a	0.1±0.5a	0.0±0.3b	0.2±0.9a	0.1±0.6a	0.0±0.1b
Lepidoptera	0.0±0.3a	0.1±0.4a	0.0±0.0b	0.2±0.6a	0.1±0.4a	0.0±0.2b
Mantodea	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0b	0.0±0.0a
Neelipleona	0.0±0.8a	0.0±0.7a	0.0±0.0b	0.2±1.8a	0.1±1.2a	0.0±0.1b
Neuroptera	0.0±0.0a	0.0±0.0b	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
Orthoptera	0.0±0.1a	0.0±0.1a	0.0±0.1a	0.0±0.1b	0.0±0.1a	0.0±0.1a
Pauropoda[1]	0.1±0.6a	0.1±0.6a	0.0±0.0b	0.5±1.3a	0.1±0.7a	0.1±0.5b
Poduromorpha	20.7±201.7b	44.3±316.2a	43.8±312.8a	8.0±21.0b	56.6±346.5a	17.3±193.0b
Protura[1]	0.0±0.1a	0.0±0.1a	0.0±0.0b	0.0±0.1a	0.0±0.0b	0.0±0.1a
Psocoptera	0.0±0.2a	0.0±0.1a	0.0±0.1b	0.0±0.3a	0.0±0.1b	0.0±0.2a
Strepsiptera	0.0±0.0a	0.0±0.0a	0.0±0.0b	0.0±0.0a	0.0±0.0a	0.0±0.0a
Symphyla[1]	0.8±3.3a	0.9±3.3b	0.0±0.1b	4.8±6.6a	1.2±3.9a	0.8±3.2b
Symphypleona	0.0±0.2b	0.1±2.4a	0.1±1.9a	0.0±0.2b	0.2±2.4a	0.0±0.1b
Thysanoptera	0.0±0.2a	0.0±0.5a	0.0±0.2b	0.1±0.8a	0.0±0.4b	0.0±0.4a
Thysanura	0.0±0.0a	0.0±0.0a	0.0±0.0b	0.0±0.0a	0.0±0.0a	0.0±0.0a
Unidentified	0.0±0.3a	0.0±0.3a	0.0±0.0b	0.2±0.7a	0.1±0.5a	0.0±0.0b

[1]Taxonomic Class (Including for analysis)

For each row, means followed by different letters are statistically different at P<0.05 (Tukey-Kramer test for multiple comparisons)

The same as for the pitfall traps, the class Collembola and the order Hymenoptera were examined in more taxonomic detail. For the class Collembola, four orders were identified only genus Cyphoderus was exclusive for this sampling type (Table 1.18.1.5). Over both cycles of evaluation (2003 and 2004), only Entomobryomorpha exhibited statistical differences among treatments, being more abundant in conventional cotton (DP 5415) (Table 1.18.1.4). The order Hymenoptera represented 5.8% of total individuals captured, with 47.8% representing the family Formicidae where 54.2% were captured in DP5415 (Table 1.18.1.4).

During two evaluation cycles, the area under the curve showed significant differences in favor of the conventional cotton for Acari and Entomobryomorpha. Only Acari exhibited changes of a cycle to other, accumulating more area during the 2004 in modified cotton.

Arthropod Taxonomic Diversity: The species richness and Shannon indices were not significantly different between the treatments NuCotn 33B and DP 5415. The Simpson index showed dominance for

one species, presenting values of 0.65 and 0.66 for DP5415 and NuCotn 33B, respectively (Table 1.18.1.6).

Table 1.18.1.4. Number of individuals and composition of arthropod orders caught in Pitfall traps and Berlese funnels in cotton, during 2003 and 2004 in the Cauca Valley, Colombia.

	Pitfall traps				Berlese funnels			
	DP 5415	**NuCotn**	**Total**	**%**	**DP 5415**	**NuCotn**	**Total**	**%**
Acarina	59,668a	63,693a	123,361	21.5	235,584a	222,015b	457,599	77.2
Aranae	468a	555a	1,023	0.2	73a	55a	128	0.0
Blattaria	44a	36a	80	0.0	13a	7a	20	0.0
Chilopoda[1]	8b	20a	28	0.0	784	666b	1,450	0.2
Coleoptera	615a	641a	1,256	0.2	1,722a	1,483b	3,205	0.5
Dermaptera	19a	24a	43	0.0	7a	6a	13	0.0
Diplopoda[1]	61a	72a	133	0.0	321a	350a	671	0.1
Diplura[1]	0	0	0	0.0	907a	821a	1,728	0.3
Diptera	343a	335a	678	0.1	748a	546b	1,294	0.2
Entomobryomorpha	4,946a	5,841a	10,787	1.9	35,903a	29,749b	65,652	11.1
Hemiptera	104a	94a	198	0.0	40a	25a	65	0.0
Homoptera	3,025a	2,903a	5,928	1.0	1,140a	961a	2,101	0.4
Hymenoptera	70,603a	64,877a	135,480	23.6	16,646a	17,877a	34,523	5.8
Isopoda	48b	113a	161	0.0	165a	134a	299	0.1
Lepidoptera	55a	49a	104	0.0	205a	217a	422	0.1
Mantodea	3a	2	5	0.0	0a	1a	1	0.0
Neelipleona	0	0	0	0.0	238a	173a	411	0.1
Neuroptera	8a	1b	9	0.0	1a	0a	1	0.0
Orthoptera	65a	50a	115	0.0	4a	7a	11	0.0
Pauropoda[1]	0	0	0	0.0	429a	389a	818	0.1
Poduromorpha	114,462b	180,067a	294,529	51.2	6,792a	7,088a	13,880	2.3
Protura[1]	0	0	0	0.0	14a	12a	26	0.0
Psocoptera	42a	22b	64	0.0	40a	35a	75	0.0
Strepsiptera	0	0	0	0.0	1a	0a	1	0.0
Symphyla[1]	30a	27a	57	0.0	4,558a	3,674b	8,232	1.4
Symphypleona	89b	525a	614	0.1	29a	22a	51	0.0
Thysanoptera	87a	70a	157	0.0	47a	72a	119	0.0
Thysanura	0	0	0	0.0	1a	1a	2	0.0
Unidentified	2a	2a	4	0.0	155a	161a	316	0.1
Sum	**254,795**	**320,019**	**574,814**	**100**	**306,567**	**286,547**	**593,114**	**100**

[1]Taxonomic Class

For each row, means followed by different letters are statistically different at P<0.05 (Tukey-Kramer test for multiple comparisons)

Finally, in the comparison of diversity between the two cycles of evaluation (2003 and 2004), no significant differences were observed in richness based on the taxa identified. The Simpson index did not differ between the two cycles of evaluation. The value of the equality index showed a tendency for greater diversity in the surveys conducted in 2003 (Table 1.18.1.6).

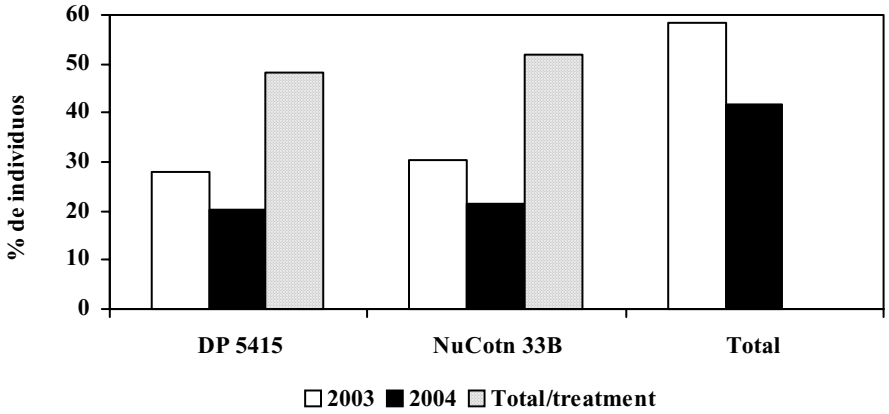

Figure 1.18.1.5. Total abundance of arthropods captured by treatment, during 2003-2004 in the Cauca Valley, Colombia.

Table 1.18.1.5. Collembola families collected from pitfall traps and berlese funnels in cotton, during 2003 and 2004 in the Cauca Valley, Colombia.

Order	Family	Genus
	Hypogasturidae	*Ceratophysella*
Poduromorpha	Brachystomellidae	*Brachystomella**
	Neanuridae	*Arlesia*
	Cyphoderidae	*Cyphoderus***
	Entomobryidae	*Seira, Lepidocyrtus*
Entomobryomorpha		*Isotoma, Proisotoma,*
	Isotomidae	*Folsomides*
	Paronellidae	*Paronella, Salina*
Symphypleona	Dicyrtomidae	*Calvatomina*
Neelipleona		

*Only on the soil surface (pitfall traps)
**Only in soil (soil cores)

Table 1.18.1.6. Indices of arthropod taxonomic (ordinal level), caught in Pitfall traps and Berlese funnels in cotton, during 2003 and 2004 in the Cauca Valley, Colombia.

Index	Between treatments		Between cycles	
	NuCotn 33B	DP 5415	2003	2004
Species richness (S)	29 a	28 a	28 a	28 a
Equity	0.40 a	0.40 a	0.42 a	0.28 a
Shannon index	1.33 a	1.32 a	1.40 a	0.96 a
Simpson index	0.65 a	0.66a	0.71 a	0.44 a

For each row, means followed by different letters are statistically different at P<0.05 (Tukey-Kramer test for multiple comparisons)

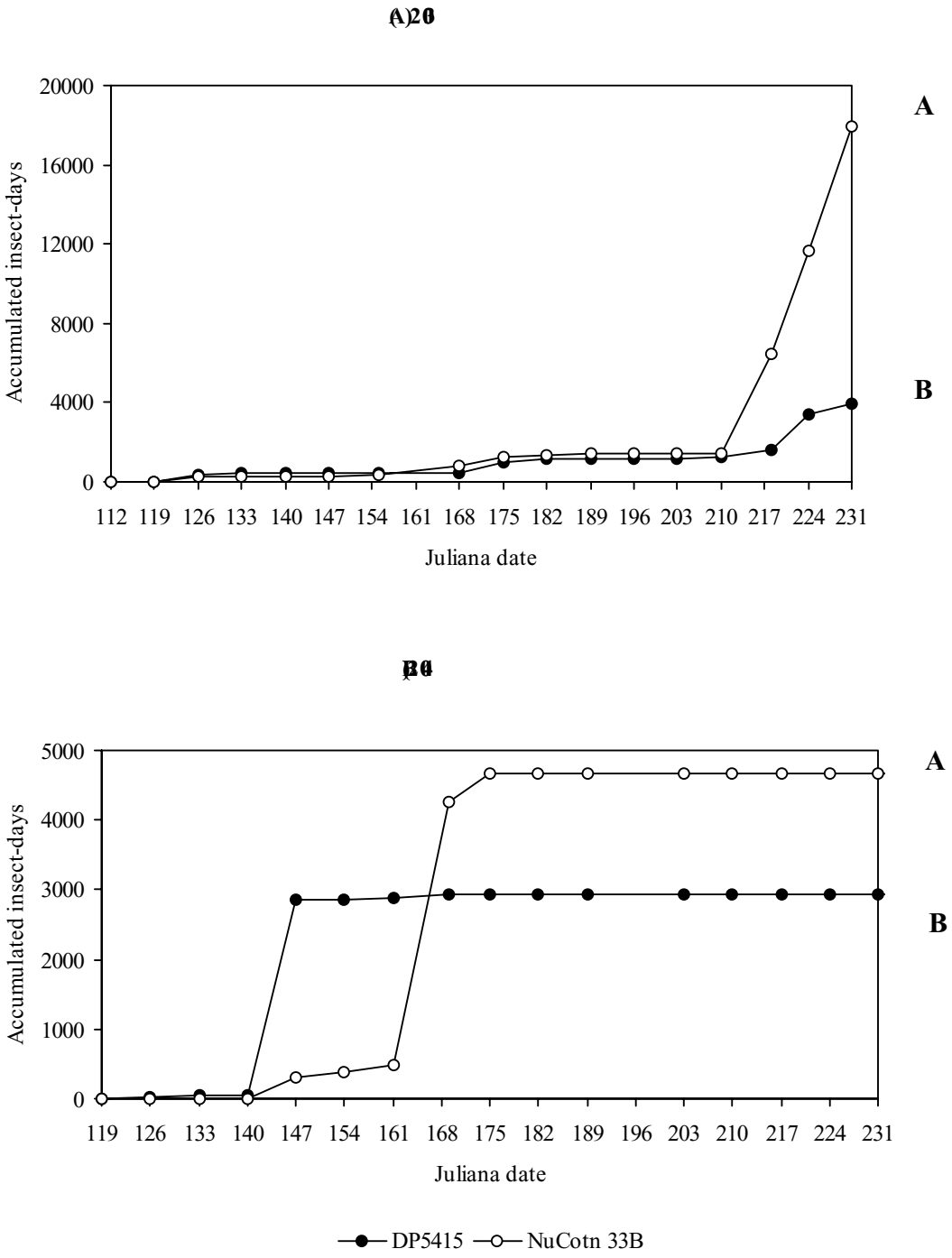

Figure 1.18.1.6 Area under the abundance curve for Poduromorpha caught in pitfall traps in cotton, during (A) 2003 and (B) 2004 in the Cauca Valley, Colombia.

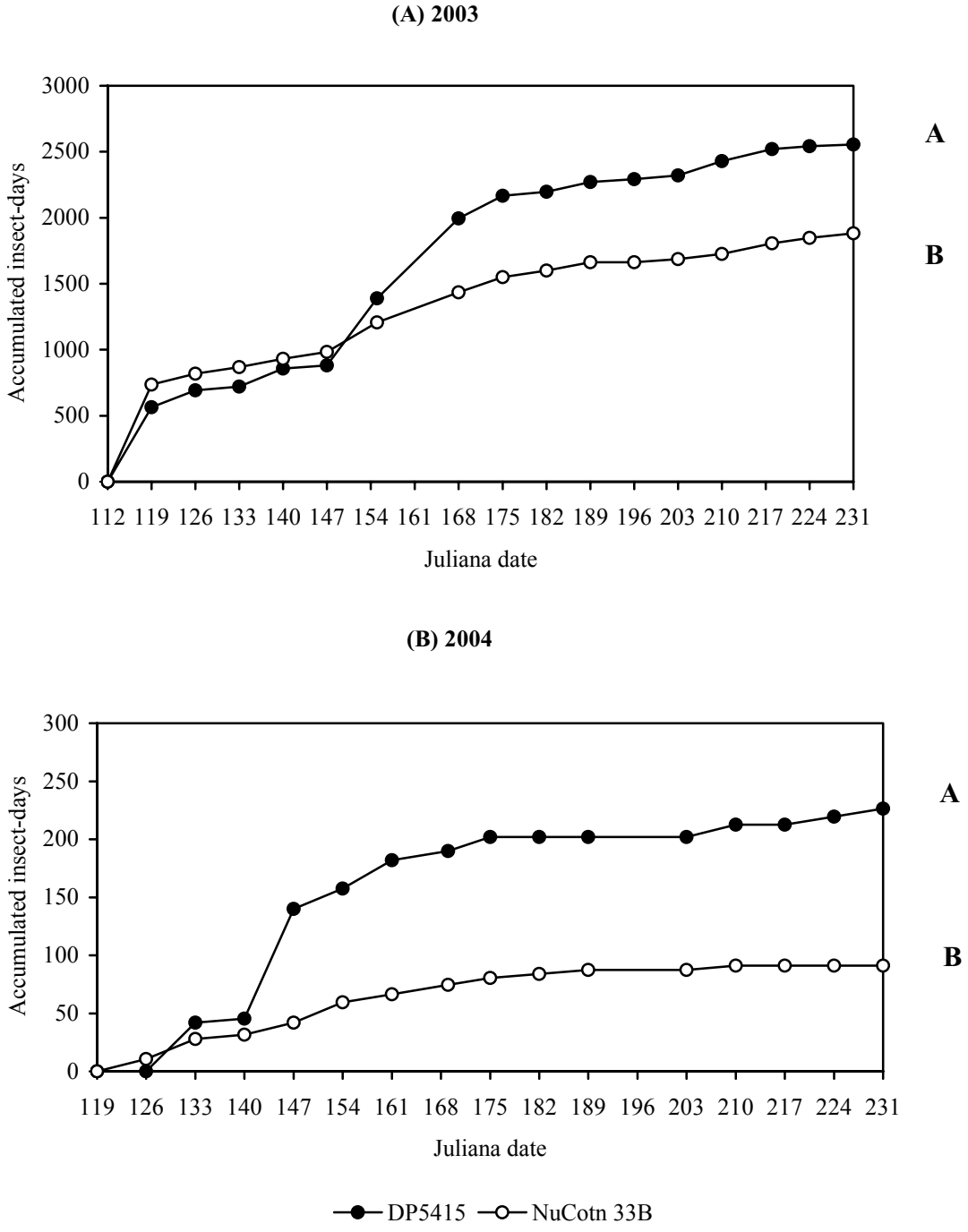

Figure 1.18.1.7. Area under the abundance curve for Hymenoptera caught in pitfall traps in cotton, during (A) 2003 and (B) 2004 in the Cauca Valley, Colombia.

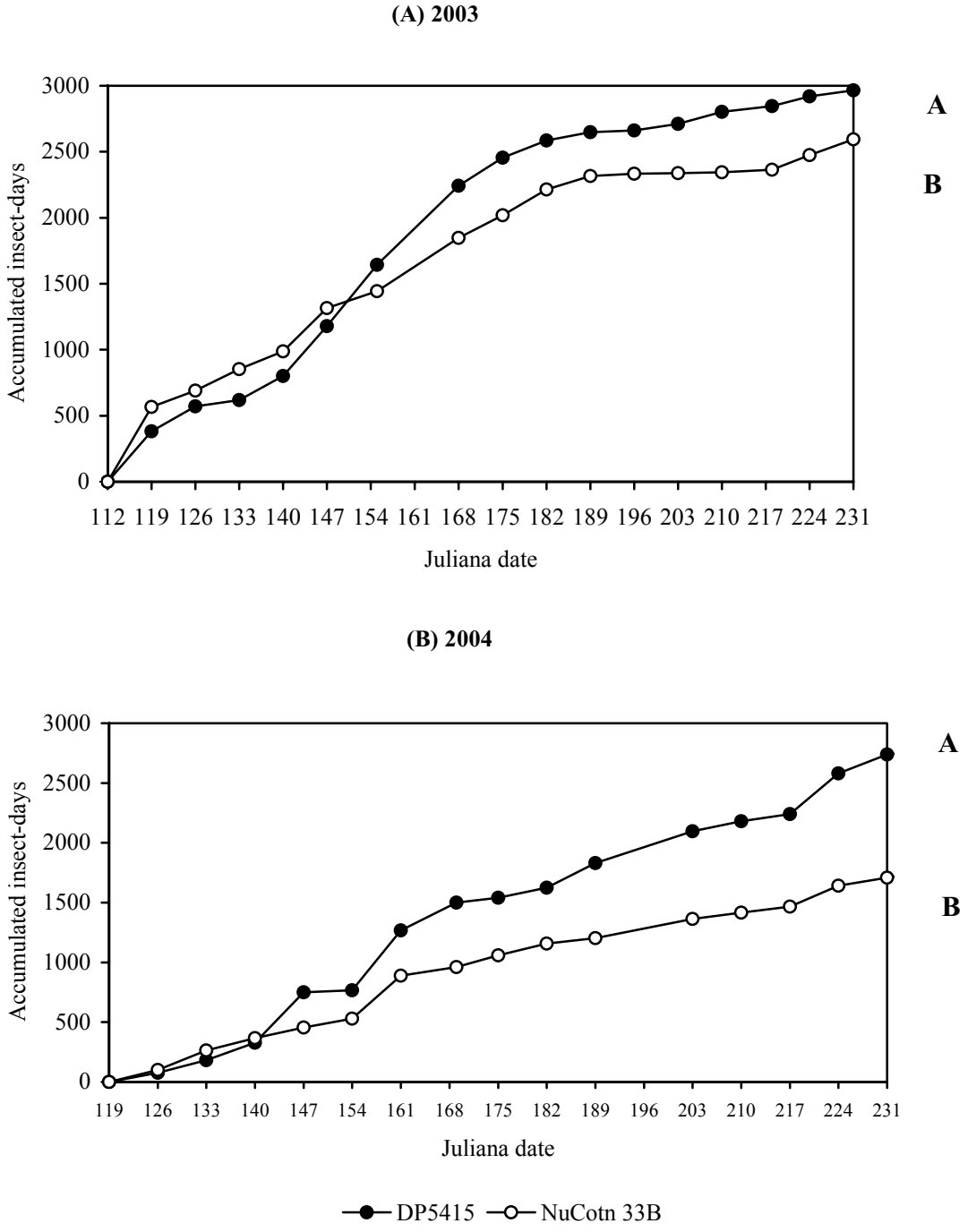

Figure 1.18.1.8. Area under the abundance curve for Acarina caught in pitfall traps in cotton, during (A) 2003 and (B) 2004 in the Cauca Valley, Colombia.

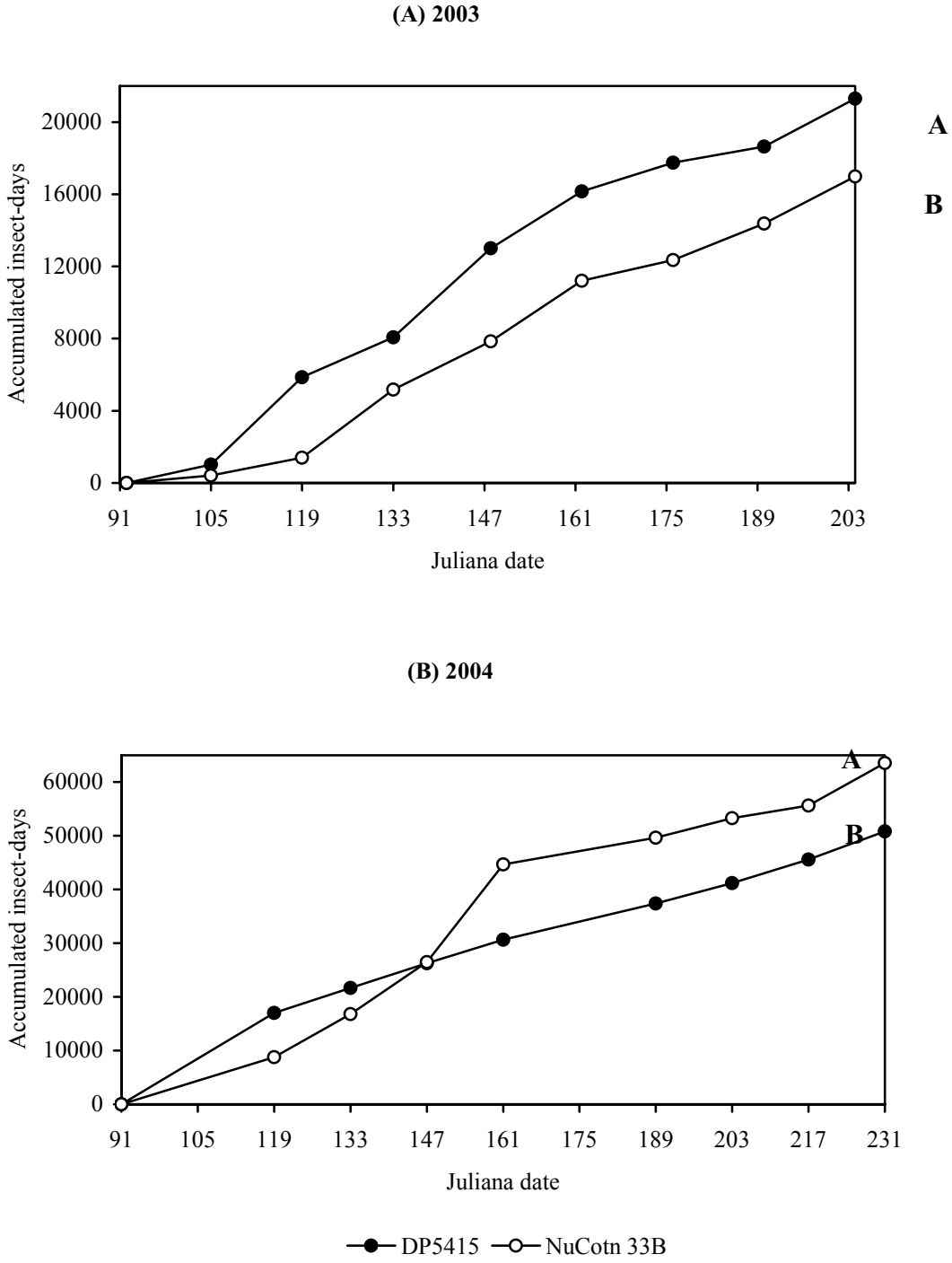

Figure 1.18.1.9. Area under the abundance curve for Acari caught in Berlese funnels in cotton, during (A) 2003 and (B) 2004 in the Cauca Valley, Colombia.

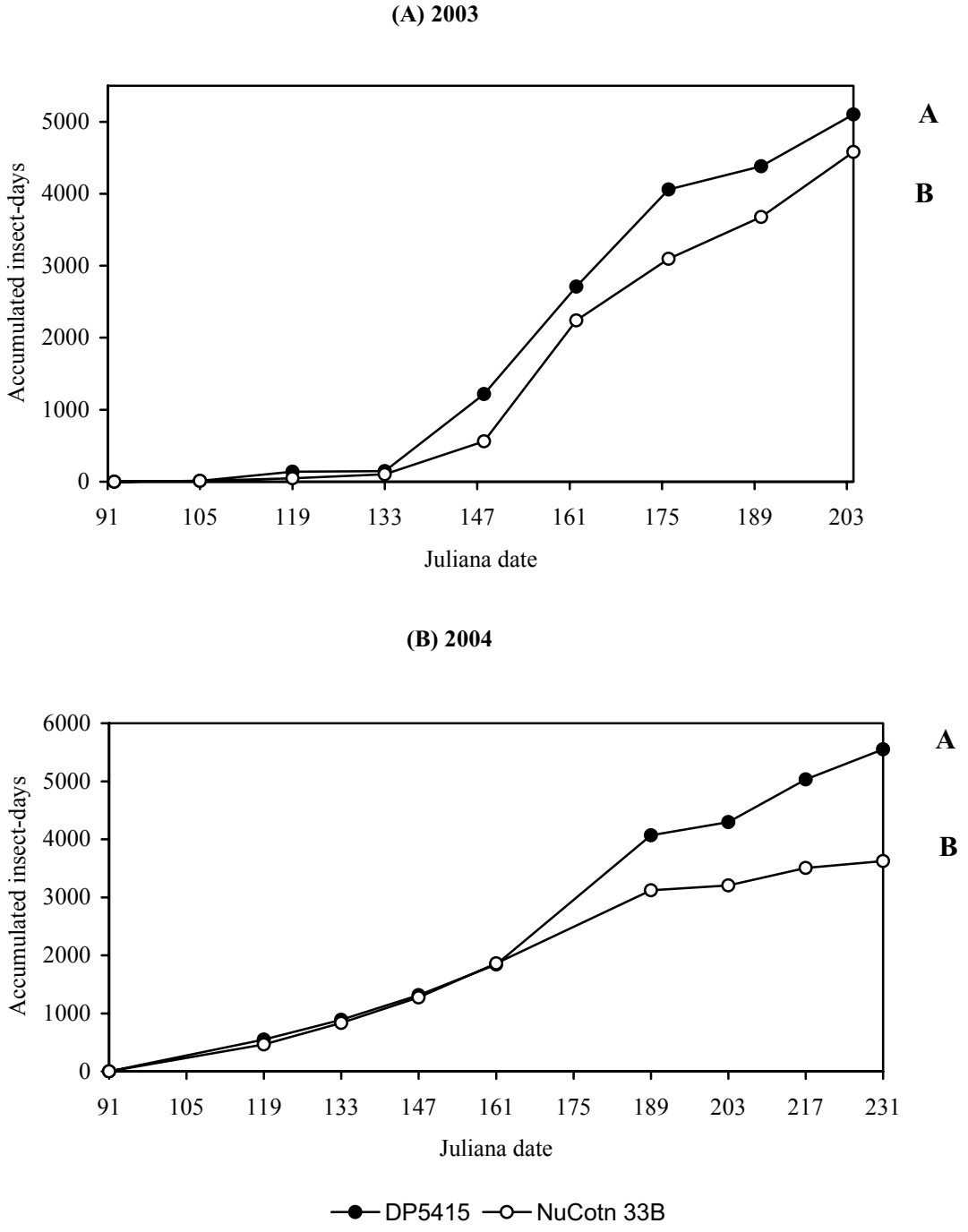

Figure 1.18.1.10. Area under the abundance curve for Entomobryomorpha caught inberlese funnels in cotton, during (A) 2003 and (B) 2004 in the Cauca Valley, Colombia.

Conclusions:

- These studies have identified a high abundance and diversity of soil-active and surface-active fauna associated with the cotton crop under the conditions of the Cauca Valley, Colombia.
- Pitfall traps are an appropriate method for measuring the abundance of surface-active arthropods and comparing their activity and diversity across treatments.
- Extracting soil cores with berlese funnels is an adequate method for measuring the abundance of soil-active arthropods and comparing their activity and diversity across treatments.
- Of the 28 taxa identified during these first two evaluation cycles in pitfall traps and Berlese funnels, only 6 exhibited statistical differences between treatments.
- Five of the identified taxa did not exhibit a significant difference in abundance between cycles (2003-2004) [Figures 1.18.1.6-10]
- Poduromorpha and Acarina were the most abundant groups in pitfall traps and Berlese funnels, respectively.
- There were no differences between treatments or evaluation cycle for any of the diversity indices evaluated.
- The abundance differences observed between treatments in the first two cycles of cotton should be studied in more detail to define how GMOs affect those differences. The protocols established in the two first cycle will therefore be implemented in three two additional cycles to better describe abundance effects over time, and to gather information to compare differences in species composition of key groups such as the springtails and ants.
- Although abundance and diversity differences may exist in response to GMO technology, it is important to determine whether the magnitude of those differences is ecologically relevant, i.e. have an effect on ecological function or overall soil health.

Activity 1.18.2. Evaluation of the ant community (Hymenoptera: Formicidae) in conventional (DP5415) and Bt (NuCotn 33B) cotton in the Cauca Valley, Colombia.

Contributors: M. Ramírez, J. Rodríguez, A. Mazo, D. C. Peck, A. C. Bellotti.

Rationale

Among the diverse technologies that have been developed with genetically modified crops is theexpression of insecticidal proteins from the bacteria *Bacillus thurigiensis* (Bt), whose target is principally larval lepidopterans. Although it has been argued that the technology is specific to this group of insects, the environmental benefits and risks have been widely argued (Brill, 1985, Science 227: 381-384 ; Colwell *et al*., 1985, Science 229: 111-112 ; Harlander,1990, Cereal Foods World 35: 1106-1109; Boulter, 1993, Phytochemistry 34: 1453-1466;Nottingham, 1998, Eat your genes, Choice Books, Marrickville, New South Wales; Porter, 1999,The good news about GM foods. Intellectual Capital 10/21/99 [On line]; Raybould *et al.,* 1999, New Phythology 141: 265-275; Barton & Dracup, 2000, Agronomy Journal 92: 797-803; NAS 2002 Environmental effects of transgenic plants: The scope and adequacy of regulation.[On line]). Since the plant contains Bt, it can be ingested by nontarget arthropods or the residues and exudates of the plant can be liberated in the soil where they can come into direct contact with nontargets (Shelton *et al*., 2002, Annual Review Entomology 47: 845-881) and generate a potential toxicity (Conner *et al*., 2003, The Plant Journal 33: 19-46). On the other hand, the indirect impact on natural enemies has also been considered given the multrophic relations in which enemies are immersed when they consume prey, parasitize hosts or consume their excretions (Dively & Rose, 2002,

1st. Int'l Symposium on Biological Control of Arthropods : 265-274: Dutton *et al*., 2003, Biocontrol 48: 661-636).

The diverse studies that have been conducted to determine the possible effects of Bt-modified crops on arthropods have focused primarily on springtails, beetles (Carabidae) and spiders. Very few have taken into account the ant community.(Dively & Rose 2002, 1st. Int'l Symposium on Biological Control of Arthropods, pp 265-274; Brooks *et al*., 2003, Philosophical Transactions of Royal Society London 358:1847–1862; Haughnton *et al*., 2003, Philosophical Transactions of Royal Society London 358: 1863–1877).

Ants are one of the most conspicous and versatile groups in agroecosystems, considering that they are numerically abundant and fulfill a great number of ecological functions, such as when they (a) associate with other groups of organisms, (b) take sugar substances from homopterans and extrafloral nectaries, (c) consume vegetative material and weed seeds, and (d) prey on other groups of arthropods (Way & Khoo 1992, Annual Rev. of Entomology 37: 479-503; Philpott *et al*., 2004, Oecologia 140: 140-149; Armbrecht *et al*., 2005, Conservartion Biology 19: 897-907).

On the other hand, ants are important components in the transformation of soil when they modify the physical and chemical conditions by incorporating organic matter and nutrients (Folgarait, 1998, Biodiversity and Conservation 7: 1221-1244). Studies that have been conducted to date on the behavior of the ant community in Bt-modified crops have not demonstrated any observable effect on this group in particular (Candolfi *et al*., 2004, Biocontrol Science and Technology 14: 129-170). One possible limitation of these studies is the time factor and it would be highly desirable to conduct longer term studies to establish whether there are effects over time. Such studies with ants have only been conducted in Bt-modified maize (Candolfi *et al*., 2004, Biocontrol Science and Technology 14: 129-170) and little emphasis has been given to other crops as evidenced by the absence of information on this group and its behavior in genetically modified crops.

Materials and Methods

This work was based on specimens collected during two years of evaluation (2003 and 2004), in the ICA research station in Palmira, located at 03°31'N, 76°19'W, 975 m elevation, annual precipitation 1295 mm, mean temperature 24°C, relative humidity 76%, and corresponding to the Holdridge life zone of Dry Tropical Forest. Specimens were collected as part of the activities of the project **"**Evaluating the Impact of Biotechnology on Biodiversity: Effect of Transgenic Maize on Non-Target Soil Organisms" where two treatments of conventional cotton (DP 5415) a Bt-transgenic Bollgard® cotton (NuCotn 33B) were evaluated (Activity 1.18.1).

Ants ere separated from the original samples of arthropods that had been collected from cotton during 2003 and 2004 and stored in 70% ethyl alcohol. This was performed by examining the samples under a stereoscope, separating all ants, and returning the remaining arthropods to their original specimen vial.

Analysis of information: To determine whether there were treatment differences in the abundance, richness and composition of the ant community, an ANOVA was performed with a randomized block design followed by the Tukey-Kramer GLM multiple comparison test at $P>0.05$. All analyses were done with the software package SAS 8.1 for Windows (SAS Institute 2000). In addition, estimates and predictions of species richness were calculated on the richness of ant species with the nonparametric Chao 2 using the specially designed software of Colwell (Colwell, 2005, Statistical Estimation of Species Richness and Shared Species from Samples. http://viceroy.eeb.uconn.edu/estimates [on line]). Data on richness in each site were randomized 100 times in order to minimize the sampling error and the

heterogeneity among sampling units (Colwell & Coddington, 1994, Philosophical Transactions of Royal Society London 345: 101-118).

Results and Discussion

Taxonomic composition: Over the two cycles of evaluation a total of 151,528 ants were captured, 94.8% corresponding to to captures in 2003. As a result of evaluations in the first cycle (2003), during 2004 the family Formicidae was included as a group of interest and we managed to identify 18 genera grouped in five subfamilies (Table 1.18.2.1). In 2004 the subfamily Myrmycinae was the most represented with 95.0% of the total capture during that period. In this subfamily the species *Wasmannia auropunctata* was the most abundant with 74.4% (Table 1.18.2.1).

Pitfall traps: For ants captured in pitfall traps, in 2004 we identified 15 genera distributed in five subfamilies (Table 1.18.2.2). Among these subfamilies the species *Wasmannia auropunctata* was the dominant species in the surveys with 93.3% of the total ants captured. This results could be explained by the extremely agressive interspecific behavior of this species influencing the low number of individuals in the other populations.

Atta cephalotes was second most abundant species, observed forraging on cotton plants and indicating that this species is feeding to some degree on Bt-modified cotton. The data show no impact on its populations due to an affect of the the Cry toxin. Ants from the genus *Solenopsis* exhibited statistically significant difference between treatments, being more abundant in Bt-modified cotton. This group consists of a large number of species that are generally omnivorous and they did not evidence a detrimental effect on their abundance as a result of the Bt-modified cotton (Table 1.18.2.2).

Berlese funnels: In the family Formicidae, five subfamilies and 13 genera were identified of which seven were more abundant in conventional cotton (Table 1.18.2.3). In addition, the most important groups in terms of abundance were the omnivorous ants of the genera *Wasmannia* and *Solenopsis*.

Taxonomic Diversity: Over the two consecutive cropping cycles, 845 samples of ants were taken, 446 in modified cotton (NuCotn 33B) and 399 in conventional cotton (DP 5415) (Figure 1.18.2.1). The captures represent five subfamilies, 20 genera and 29 different species. Of the 29 species identified, 24 were common to both modified and conventional cotton, while three were exclusive to modified cotton and one to conventional cotton.

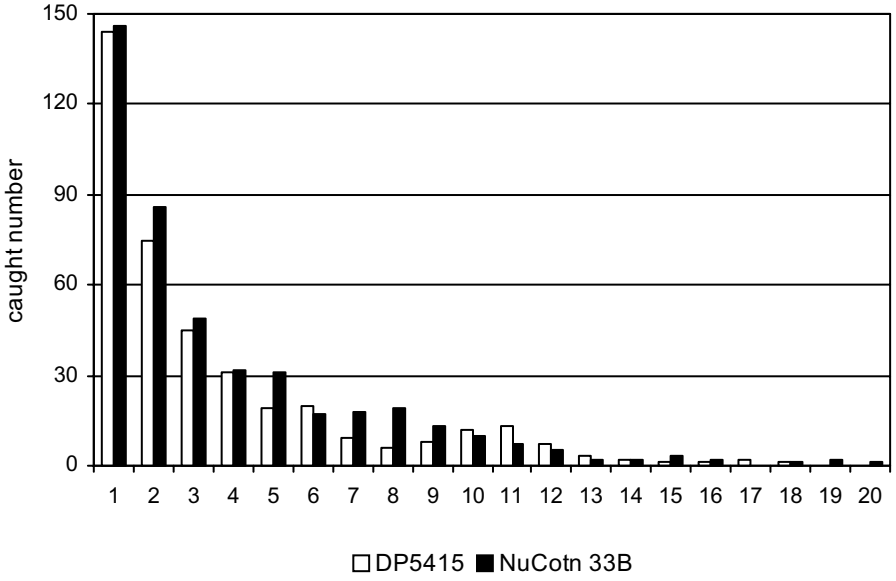

Figure 1.18.2.1. Distribution of the ant genera found in Bt-modified and conventional cotton in experimental plots at ICA, Palmira. 1 = *Wasmannia*, 2 = *Solenopsis*, 3 = *Hypoponera*, 4 = *Monomorium*, 5 = *Ectatomma*, 6 = *Rogeria*, 7 = *Atta*, 8 = *Strumigenys*, 9 = *Pheidole*, 10 = *Brachymyrmex*, 11 = *Cyphomyrmex*, 12 = *Paratrechina*, 13 = *Tapinoma*, 14 = *Neivamyrmex*, 15 = *Tranopelta*, 16 = *Probolomyrmex*, 17 = *Cardiocondyla*, 18 = *Nomamyrmex*, 19 = *Pachycondyla*, 20 = *Linepithema*.

Estimate of richness: Based on the species richness estimate Chao2 (calculated from the presence/absence of species), according to the survey methods implemented in the study, 93.0 y 89.0% of the species were captured in modified and conventional cotton, respectively (Figure 1.18.2.2A and 1.18.2.2B). This effort is considered to have identified a representative richness of ants, taking into account that the curve stabilized in the Bt-modified cotton and tended toward stabilization in the conventional cotton. The same result is complemented by the behavrior of the curves of the "uniques" (species that occur in only one sample) and the "duplicates" (species that occur in two samples which converge before arriving at the end of the two years of evaluation. When the singleton and doubleton curves converge it means that the sampling effort has been sufficiently complete to describe the richness present. McKamey (1999, American Entomologist 45: 213-222) indicates that in inventories of groups of insects, the amount of time and effort invested does not matter because it is possible to never arrive at the real number of species, and therefore these species richness estimates become useful tools.

Richness and abundance: For these indices, no differences were detected between Bt-modified and conventional cotton for the richness and abundance of ants. This is unlike what was observed in comparing abundance between years, where there were differences in richness and abundance. The differnce between years could be explained b a prolonged period of drought during 2004 which may have influenced the number of captures and the ant populations (Figure 1.18.2.3).

Ant guilds: The ant fauna from the cotton treatments were grouped into functional guilds for further analysis. This classification refers to groups of species that obtain their subsistence from the same types of resources and use the same strategies in occupying their ecological niches. In this study, they were grouped into three guilds taking into account the number of occurrences, capture method and based on the

bibliographic references of Andersen (2000, *In*: Agosti D., J.D. Majer, L.E. Alonso, T.R. Schultz (eds.), Standard methods for measuring and monitoring biodiversity, pp 25-34) and Silvestre *et al.* (2003, In: Fernández, F (ed.). Introducción a las hormigas de la región Neotropical. Instituto de Investigaciones Entomológicas Alexander von Humboldt y Smithsonian Institution. Bogotá, Colombia. 424)

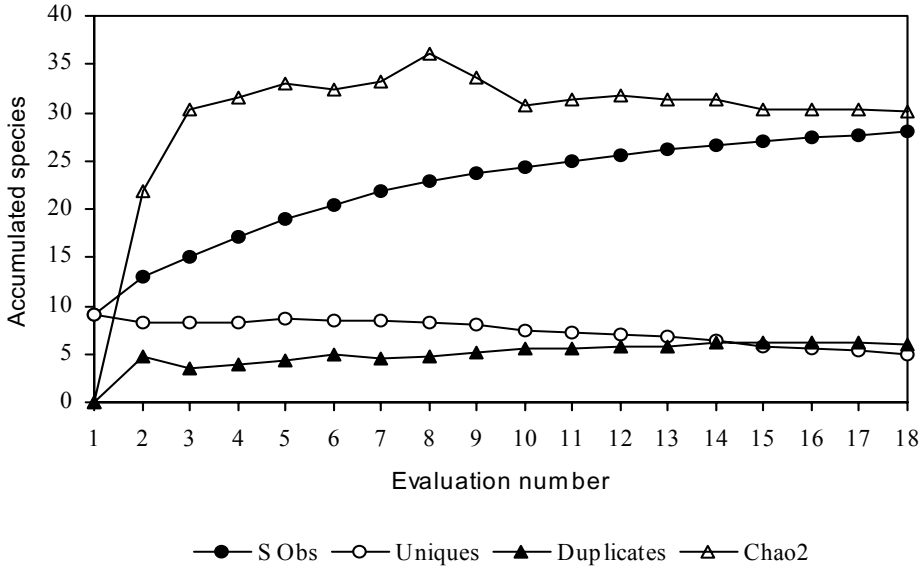

Figure 1.18.2.2A. Species accumulation curves over two years of evaluation in Bt-modified cotton. ICA, Palmira, Valle del Cauca. (Dates 1-9 are 2003, 10-18 are 2004).

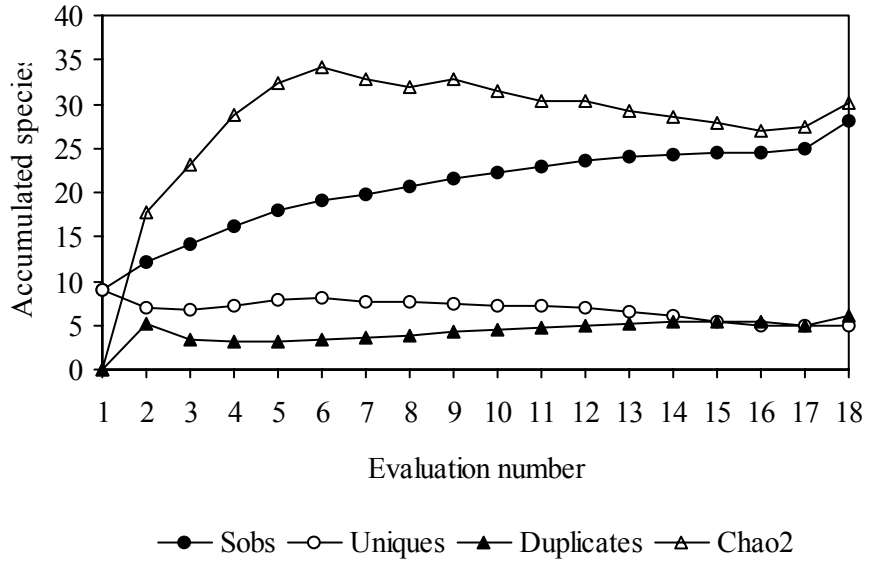

Figure 1.18.2.2B. Species accumulation curves over two years of evaluation in conventional cotton. ICA, Palmira, Valle del Cauca. (Dates 1-9 are 2003, 10-18 are 2004).

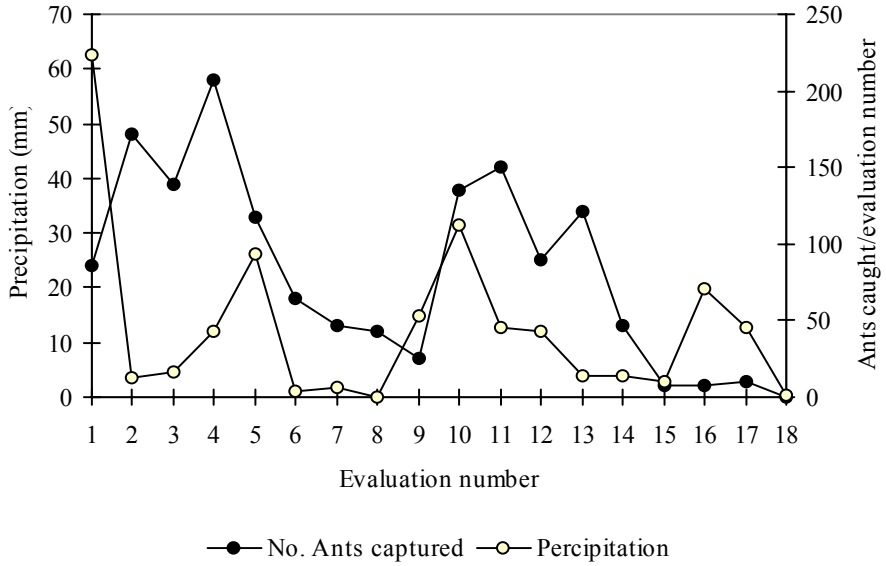

Figure 1.18.2.3. Accumulated weekly Precipitation during 2003-2004 versus the number of ants captured.

1. Omnivores –the majority of ant species; function as opportunistic generalists in their diet; include genera such as *Wasmannia*, *Solenopsis* and *Ectatomma*.
2. Cryptics – represented by species of small size; very few captures in the samples.
3. Specialized cryptic predators – small species that live in the litter; manibles are well-developed for specialized diets that include springtails and diplurans; includes species of the genus *Strumigenys*.

An assessment of guilds will help us understand the structure and function of the ant community according to vegetation and land-use patterns, among others (Andersen, 2000, In: Standard methods for measuring and monitoring biodiversity, 280 p; Silvestre *et al.*,.2003, In : Fernández, F (ed.). Introducción a las hormigas de la región Neotropical. Instituto de Investigaciones Entomológicas Alexander von Humboldt y Smithsonian Institution. Bogotá, Colombia. 424), and in this case the possible impact that could be related to Bt-modified cotton on the soil arthropod communities. In a comparison between treatments, the only significant difference occurred in the specialized cryptic predators, being more abundant in Bt-modified cotton. The possible causes for this include microclimate, distribution of food sources, and nesting resources but not possible exposure to the Cry toxin. Analyses of other insect orders have shown this same tendency. In the case of Coleoptera, Mazo *et al*. (2005, Resumenes XXXII Congreso SOCOLEN, Colombia, 88 p.) found that of 22 families captured, two (Cicindellidae and Lathrididae) were statistically more abundant in Bt-modified cotton than conventional cotton. Similarly, for the class Collembola, the orders Poduromorpha and Entomobryomorpha were more abundant in Bt-modified cotton (Mazo 2005, Efecto del algodón Bollgard ® (Bt) sobre la diversidad y abundancia de artrópodos del suelo durante su segundo año en el Valle del Cauca. Undergraduate Thesis. Universidad del Valle, Facultad de Ciencias, Cali. 67 p)

Conclusions:

∉ Over the two cycles of evaluation in conventional and Bt-modified cotton, no differneces were detected in the ant community. Therefore this study does not implicate the Cry toxin as having any effect on this community. It remains to be seen whether effects may accumulate over th elonger term and therefore

studies should be conducted over consecutive years as complementary tests of possible effects on the soil arthropod community.

∉ It is possible to conduct evaluations in this manner to establish the impact of the introduction of Bt-modified crops on the community of arthropods associated with them, not just soil communities, but foliar communities.

∉ This study offers basic information on how to establish the risk of introduction of genetically modified crops in the Cauca Valley. Nevertheless, this only consititutes a fraction of the full range of organisms present in the cotton crop and of the interspecific plant-arthropod interactions that can occur within. It is necessary to conduct further studies on this community and on other groups of organisms.

Table 1.18.2.1. Comparison of the abundance (number and percentage) of captured ants in pitfall traps and soil samples, during 2004, in C.I. del ICA - Palmira, in cotton.

Taxa	Pitfall traps		Soil samples		Total	
	No. Individuals	%	No. Individuals	%	No. Individuals	%
Myrmycinae						
Wasmannia auropunctata	4.448	93.3	1.330	44.3	5.778	74.4
Solenopsis	93	2.0	980	32.7	1.073	13.8
Monomorium	10	0.2	346	11.5	356	4.6
Pheidole	61	1.3	75	2.5	136	1.8
Atta cephalotes	30	0.6	0	0	30	0.4
Cardiocondyla	2	0.0	1	0.0	3	0.0
Cyphomyrmex rimosus	2	0.0	0	0	2	0.0
Strumigenys	1	0.0	1	0.0	2	0.0
Tranopelta	1	0.0	0	0	1	0.0
Formicinae						
Brachymyrmex	6	0.1	20	0.7	26	0.3
Camponotus	0	0	1	0.0	1	0.0
Paratrechina	6	0.1	1	0.0	7	0.1
Dolichoderinae						
Tapinoma melanocephalum	4	0.1	24	0.8	28	0.4
Ecitoninae						
Nomamyrmex esenbeckii	3	0.1	0	0	3	0.0
Neyvamyrmex	0	0	1	0.0	1	0.0
Ponerinae						
Probolomyrmex	0	0	3	0.1	3	0.0
Hypoponera	91	1.9	165	5.5	256	3.3
Ectatoma tuberculatum	10	0.2	0	0	10	0.1
Winged Formicidae	0	0	51	1.7	51	0.7
Total	**4.768**	**100**	**2.999**	**100**	**7.767**	**100**

For each row, values followed by distinct letters are statistically different (P<0.05) (Data transformed Ln(x+1), Tukey Test and GLM for multiple comparisons).

Table 1.18.2.2. Comparison of the abundance (number and percentage) of captured ants in conventional (DP5415) and Bt-modified (NuCotn 33B) cotton during 2004in C.I. del ICA, Palmira, from pitfall traps.

Taxa	DP5415		NuCotn 33B		Total	
	No. Individuals	%	No. Individuals	%	No. Individuals	%
Myrmycinae						
Wasmannia auropunctata	1.277 b	90,2	3.171 a	94,6	4.448	93,3
Solenopsis	31 b	2,2	62 a	1,	93	2,0
Pheidole	25 a	1,	36 a	1,	61	1,3
Atta cephalotes	7 b	0,	23 a	0,	30	0,6
Monomorium	9 a	0,	1 a	0,	10	0,2
Cardiocondyla	1 a	0,	1 a	0,	2	0,0
Cyphomyrmex rimosus	1 a	0,	1 a	0,	2	0,0
Strumigenys	0 a	0,	1 a	0,	1	0,0
Tranopelta	0 a	0,	1 a	0,	1	0,0
Formicinae						
Brachymyrmex	5 a	0,	1 a	0,	6	0,1
Paratrechina	3 a	0,	3 a	0,	6	0,1
Dolichoderinae						
Tapinoma melanocephalum	2 a	0,	2 a	0,	4	0,1
Ecitoninae						
Nomamyrmex esenbeckii	2 a	0,	1 a	0,	3	0,1
Ponerinae						
Hypoponera	50 a	3,	41 a	1,	91	1,9
Ectatoma tuberculatum	3 a	0,	7 a	0,	10	0,2
Total	1.416	10	3.352	10	4.768	100

For each row, values followed by distinct letters are statistically different (P<0.05) (Data transformed Ln(x+1), Tukey Test and GLM for multiple comparisons).

Table1.18.2.3. Comparison of the abundance (number and percentage) of captured ants in conventional (DP5415) and Bt-modified (NuCotn 33B) cotton during 2004in C.I. del ICA, Palmira, from soil samples.

Taxa	DP5415		NuCotn 33B		Total	
	No. Individuals	%	No. Individuals	%	No. Individuals	%
Myrmycinae						
Cardiocondyla	1a	0,1	0a	0,0	1	0,0
Strumigenys	1a	0,1	0a	0,0	1	0,0
Pheidole	61a	3,5	14b	1,1	75	2,5
Monomorium	14b	0,8	332a	26,6	346	11,5
Solenopsis	543	31,0	437	35,1	980	32,7
Wasmannia auropunctata	982a	56,0	348b	27,9	1330	44,3
Formicinae						
Brachymyrmex	14a	0,8	6a	0,5	20	0,7
Camponotus	0a	0,0	1a	0,1	1	0,0
Paratrechina	0a	0,0	1a	0,1	1	0,0
Dolichoderinae						
Tapinoma melanocephalum	7[a]	0,4	17a	1,4	24	0,8
Ecitoninae						
Neyvamyrmex	0a	0,0	1a	0,1	1	0,0
Ponerinae						
Probolomyrmex	0a	0,0	3a	0,2	3	0,1
Hypoponera	106a	6,0	59b	4,7	165	5,5
Alados Formicidae	24a	1,4	27a	2,2	51	1,7
Total	1753	100	1246	100	2999	100

For each row, values followed by distinct letters are statistically different (P<0.05) (Data transformed Ln(x+1), Tukey Test and GLM for multiple comparisons)

Activity 1.18.3. Comparison of the abundance and diversity of Coleoptera in conventional (DP5415) and modified (NuCotn 33B) cotton in the Cauca Valley, Colombia.

Contributors: A. Mazo, J.Rodríguez, D. C. Peck, J. Montoya Lerma, and A. C. Bellotti.

Materials and Methods

This work was based on specimens collected during 2004, in the ICA research station in Palmira, located at 03°31'N, 76°19'W, 975 m elevation, annual precipitation 1295 mm, mean temperature 24^{o}C, relative humidity 76%, and corresponding to the Holdridge life zone of Dry Tropical Forest. Specimens were collected as part of the activities of the project "Evaluating the Impact of Biotechnology on Biodiversity: Effect of Transgenic Maize on Non-Target Soil Organisms" where two treatments of conventional cotton (DP 5415) a Bt-transgenic Bollgard® cotton (NuCotn 33B) were evaluated. (Activity 1.18.1)

Coleoptera were separated from the original samples of arthropods that had been collected from cotton during 2004 and stored in 70% ethyl alcohol. This was performed by examining the samples under a stereoscope, separating all Coleoptera, and returning the remaining arthropods to their original specimen vial.

Analysis of information: The statistical model used for the analysis of the data was a completely randomized block design. With this design an ANOVA was used to determine differences in abundance among treatments and determine the effect of their interactions.

Results and Discussion

Pitfall traps: For the order Coleoptera, in the second evaluation cycle (2004) 748 individuals were captured, distributed in 19 families. Of those families, 10 were more abundant in conventional cotton. Nevertheless, the overall abundance of beetles was 1.1 times higher in Bt-modified cotton. Of the 19 families identified, only Cicindellidae and Lathridiidae exhibited significant differneces between treatments, in both cases being more abundant in Bt-modified cotton.

The most abundant families in 2004 were Carabidae and Scarabaeidae with 36.2 and 17.3% of the total individuals captured. Among the captured carabids were representatives of the genus *Calosoma* (caterpillar hunters), *Galeritula*, *Scarites*, and other unidentified morphospecies.(Figure 1.18.3.1). In terms of abundance, *Galeritula* was the most represented (209 individualscaptured), with 51.2% of captures in conventional cotton. (Candolfi *et al.,* 2004, Biocontrol Science and Technology 14: 129 – 170; Lozzia, 1999, Bollettino di Zoologia Agraria e di Bachicoltura 31: 37-58; Haungton *et al.*, 2003, Philosophical Transactionsof the Royal Society B 358: 1863–1877; Duan *et al.*, 2004, Environmental Entomology 33:275-281; ř ezáļ *et al,* 2005, Proceedings Ecological Impact of GMOs. Int'l org. for biological and integrated control of Noxious Animals and Plants June 1-3. Lleida, Catalonia, España; Turanli *et al*., 2005, Proceedings Ecological Impact of GMOs. Int'l org. for biological and integrated control of Noxious Animals and Plants, June 1-3, Lleida, Catalonia, España) Diverse studies support the observations made on this group in Bt-modified cotton, that no effects are reported that diminish populations of these relatively abundant taxa.

In the Scarabaeidae, four subfamilies were identified (Figure 1.18.3.2). Melolonthinae (*Cyclocephala lunulata*) and Rutelinae (*Leucothyreus femuratus*) represented about 10.0% of the captured individuals. Aphodinae and Scarabaeinae (*Canthon* sp.) were the groups of greatest abundance in this family, representing approximately 90.0% of the captures. No statistical differences were detected in abundance due to treatments. This result is comparable to that reported by Frizzas (2003, MSc Thesis, Escuela superior de agricultura Janeiro, Sao Paulo, Brasil, 192 pp) in conventional and Bt-transgenic maize.

Only one species of Cicindellidae was identified, belonging to the genus *Megacephala* sp. The abundance of this species was 2.1 times higher in Bt-transgenic cotton. In Brazil, Frizzas (2003) obtained similar results with respect to cicindelids in Bt-transfgenic maize, confirming the idea that the Cry toxin does not have a negative effect on this group of predators. The family Lathridiidae could be more exposed to the Cry toxin because of their mycetophaguos habits. Nevertheless, results showed this family to be 2.7 times more abundant in Bt-modified cotton (Table 1.18.3.2)

Berlese funnels: A total of 822 individuals, distributed in 15 families, were captured from soil cores extracted with Berelese funnels. Conventional cotton had 1.3 times more captures than Bt-transgenic cotton, but a statistically significant difference was not detected (Table 1.18.3.1).

Table 1.18.3.1. Comparison of the abundance (in total number and percentage) of Coleoptera captured in pitfall traps in conventional and Bt-modified cotton, during 2004, in C.I. del ICA, Palmira.

Family	DP5415		NuCotn 33B		Total	
	No. Individuals	%	No. Individuals	%	No. Individuals	%
Carabidae	138 a	37,9	133 a	34,6	271	36,2
Scarabaeidae	63 a	17,3	66 a	17,2	129	17,3
Cicindellidae	22 b	6,0	47 a	12,2	69	9,2
Chrysomelidae	28 a	7,7	34 a	8,9	62	8,3
Nitiduliidae	35 a	9,6	22 a	5,7	57	7,6
Staphylinidae	17 a	4,7	23 a	6,0	40	5,4
Coccinellidae	10 a	2,8	16 a	4,2	26	3,5
Lathridiidae	7 b	1,9	19 a	5,0	26	3,5
Curculiionidae	10 a	2,8	8 a	2,1	18	2,4
Scydmaenidae	10 a	2,8	5 a	1,3	15	2,0
Cucujidae	10 a	2,8	4 a	1,0	14	1,9
Anthicidae	3 a	0,8	2 a	0,5	5	0,7
Corylophidae	5 a	1,4	0 a	0,0	5	0,7
Scolytidae	1 a	0,3	3 a	0,8	4	0,5
Histeridae	2 a	0,6	0 a	0,0	2	0,3
Tenebrionidae	1 a	0,3	1 a	0,3	2	0,3
Cantharidae	1 a	0,3	0 a	0,0	1	0,1
Cleridae	0 a	0,0	1 a	0,3	1	0,1
Phalacridae	1 a	0,3	0 a	0,0	1	0,1
Sum	364	100	384	100	748	100

For each row, values followed by distinct letters are statistically different ($P<0.05$) (Data transformed Ln(x+1), Tukey Test and GLM for multiple comparisons).

Of the Coleoptera families identified, Staphylinidae and Scarabaeidae were the most abundant, with 55.2 and 27.4% of captures, respectively (Table 1.18.3.1). In both cases the greatest abundance was associated with conventional cotton with 1.4 and 1.3 times more captures than the Bt-transgenic variety, respectively. In addition, the families Scolytidae and Carabidae also had relatively high captures for the order. Scolytidae was 1.2 times more abundant in conventional cotton while Carabidae was 1.7 times more abundant in Bt-transgenic cotton (Table 1.18.3.1). This information agrees with previous studies that have not identified negative effects associated with Bt-transgenic crops (Candolfi e*t al.,* 2004, Biocontrol Science and Technology 14: 129-170).

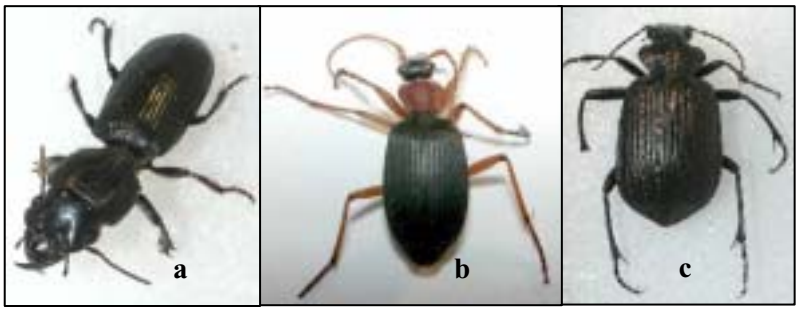

Figure 1.18.3.1. Morphospecies of the family Carabidae found in cotton in C.I. del ICA Palmira, 2004. (a) *Scarites,* (b) *Galeritula*, (c) *Calosoma*

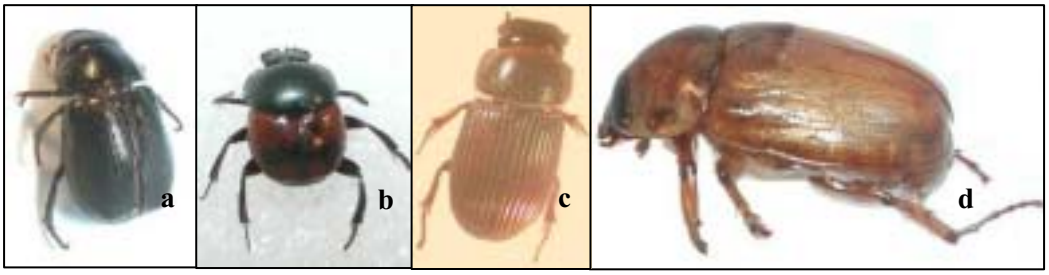

Figure 1.18.3.2. Morphospecies of the family Scarabaeidae found in cotton in C.I. del ICAPalmira, 2004. (a) Rutelinae, (b) Scarabaeinae, (c) Aphodiinae y (d) Dynastinae.

Table 1.18.3.2. Comparison of the abundance (in total number and percentage) of Coleoptera captured in soil cores extracted with Berlese funnels in conventional and Bt-modified cotton, during 2004, in C.I. del ICA, Palmira.

Family	DP5415		NuCotn 33B		Total	
	No. Individuals	%	No. Individuals	%	No. Individuals	%
Staphylinidae	268 a	57,9	186 a	51,8	454	55,2
Scarabaeidae	127 a	27,4	98 a	27,3	225	27,4
Scolytidae	35 a	7,6	30 a	8,4	65	7,9
Carabidae	9 a	1,9	17 a	4,7	26	3,2
Nitiduliidae	7 a	1,5	5 a	1,4	12	1,5
Cucujidae	5 a	1,1	4 a	1,1	9	1,1
Anthicidae	3 a	0,6	5 a	1,4	8	1,0
Curculiionidae	3 a	0,6	3 a	0,8	6	0,7
Chrysomelidae	3 a	0,6	2 a	0,6	5	0,6
Lathridiidae	1 a	0,2	3 a	0,8	4	0,5
Elateridae	1 a	0,2	2 a	0,6	3	0,4
Coccinellidae	0 a	0,0	2 a	0,6	2	0,2
Ptiliidae	0 a	0,0	1 a	0,3	1	0,1
Ptylodactylidae	1 a	0,2	0 a	0,0	1	0,1
Scydmaenidae	0 a	0,0	1 a	0,3	1	0,1
Sum	**463**	**100**	**359**	**100**	**822**	**100**

For each row, values followed by distinct letters are statistically different (P<0.05) (Data transformed Ln(x+1), Tukey Test and GLM for multiple comparisons).

Activity 1.19. Determining the genetic variability of *Ralstonia solanacearum* of plantain, using microsatellite markers (RAMs)

Contributors: E. Álvarez, E. Gómez, J. F. Mejía, G. Llano, and J. Loke

Highlight:

- The first report of the pathogen's genetic diversity as determined by RAM analysis was presented. This analysis permitted identification of infra-subspecific groups of strains that have common biological properties, evolutionary relationships, or geographic origins. *Ralstonia solanacearum* DNA sequences were reported to GenBank.

Rationale

Characterization and knowledge of the genetic structure of pathogen populations have direct applications in disease management. This study therefore aimed to obtain information on the genetic diversity of a population of *R. solanacearum* race 2 from Colombia, causal agent of bacterial wilt of plantain. For the first time a technique based on microsatellites, known as random amplified microsatellites (RAMs), has been used with this pathogen. The main objective of this study was to determine the variability of *Ralstonia solanacearum* from Musaceae crops in different regions of Colombia. Our goal is to develop strategies to improve the acquisition of durable resistance to *R. solanacearum*.

Materials and Methods

We used 59 strains of *R. solanacearum* from tissues of sick plants, infected by bacterial wilt and collected from plantain, banana, and heliconia crops growing in six departments of Colombia, and from soil of infected plantain crops in Quindío. We used, for controls, a strain from eggplant from Kenya and four strains from tobacco (two from Floridablanca, Santander, Colombia; one from Quency, Florida, USA; and one from Japan) (Table 1.19.1). The *R. solanacearum* strains were obtained from the collection held by the Cassava Pathology program at CIAT. This study thus became the most complete on the genetic variability of the causal agent of bacterial wilt of plantain in Colombia. The strains preserved in solution with 60% glycerol were reactivated in a semi-selective medium (SMSA) and then transferred to nutrient agar to obtain pure strains of 24-h growth for later DNA extraction.

Extracting DNA: The protocol described by Boucher *et al.,* (1985, J. Gen. Microbiol. 131:2449-2457) was used to extract DNA from the 59 strains.

Analyzing RAMs: To determine the genetic variability of the Ralstonia solanacearum strains, random amplification of microsatellite primers (RAMs) was used. RAM primers are tandem repeats of sequences of two or three nucleotides with random bases in the 3' extreme. With these, the genetic variability of individuals belonging to very close gene pools can be estimated, thus permitting differentiation between species and even within a single species, according to patterns of amplification from the total DNA.

We evaluated polymorphism among five isolates of R. solanacearum, obtained with seven RAM primers: HVH $(TG)_7T$, DHB $(CGA)_5$, DYD $(CT)_7C$, DBD $A(CA)_7$, VHV $(GT)_5G$, HBH $(AG)_7A$, and DDB $(CCA)_5$, where H is (A,T,C); B is (G,T,C); V is (G,A,C); and D is (G,A,T). We then amplified the 59 strains with the most polymorphic primer (Hantula *et al*., 1996, European Journal of Forest Pathology 26:159-166).

Every PCR-RAM reaction was carried out in volumes of 12.5 µL, made up of dATP, dCTP, dGTP, and

dTTP in proportions of 0.2 mM each; 1.25 µL 10X *Taq* polymerase buffer solution; 1 mM $MgCl_2$; 0.008 U *Taq* polymerase (Promega); 2 ε M primer; and 5 ng total DNA. Amplification was carried out in a MJ Research PTC-100 thermal cycler, programmed at 95 ∀C for 5 min; 37 cycles of denaturation at 95 ∀C for 30 s; annealing at 55 ∀C (TG primer), 61 ∀C (CGA), 41 ∀C (CT), 50 ∀C (CA and AG), and 58 ∀C (GT), all for 45 s, and 55 ∀C (CCA) for 50 s; extension at 72 ∀C for 2 min; and final extension at 72 ∀C for 7 min (Henríquez et al., 2002*,* Phytopathology 92:580-589).

Table 1.19.1. Description of *Ralstonia solanacearum* strains, causal agent of bacterial wilt of plantain, used for the study.

Crop and strain no.	Geographical origin	Source	Crop and strain no.	Geographical origin	Source
Plantain			**Plantain**		
1	Quindío	Rachis	73	Urabá (Antioquia)	Pseudostem
3	Quindío	Petiole	76	Montenegro (Quindío)	Pseudostem
15	Quindío	Soil	78	Montenegro (Quindío)	Rachis
16b	Quindío	Soil	79	Montenegro (Quindío)	Rhizome
17	Jamundí (Valle del Cauca)	Soil	80	Montenegro (Quindío)	Pseudostem
18	Jamundí (Valle del Cauca)	Sucker	83	Quindío	Fruit
32	Caquetá	Pseudostem	84	Quindío	Pseudostem
3	Caquetá	Pseudostem	85	Quindío	Sucker
81	Montenegro (Quindío)	Fruit	86	Calarcá (Quindío)	Rachis
38	Quindío	Soil	88	La Tebaida (Quindío)	Rhizome
39	Quindío	Soil	89	La Tebaida (Quindío)	Pseudostem
40	Quimbaya (Quindío)	Soil	97	Quimbaya (Quindío)	Rhizome
41	Quimbaya (Quindío)	Soil	107	Armenia (Quindío)	Fruit
42	Fuente de Oro (Meta)	Pseudostem	1008	Ibagué (Tolima)	CIAT collection
43	Fuente de Oro (Meta)	Pseudostem			
48	Armenia (Quindío)	Fruit	**Banana**		
54	Fuente de Oro (Meta)	Pseudostem	5	Urabá (Antioquia)	Rhizome
57	Fuente de Oro (Meta)	Pseudostem	6	Urabá (Antioquia)	Fruit
58R	Fuente de Oro (Meta)	Petiole	7	Urabá (Antioquia)	Fruit
59	Fuente de Oro (Meta)	Pseudostem	110	Magdalena	Pseudostem
60	Fuente de Oro (Meta)	Pseudostem	111	Magdalena	Rhizome
63	Granada (Meta)	Pseudostem	112	Magdalena	Sucker
64	Granada (Meta)	Pseudostem			
65	Granada (Meta)	Pseudostem	**Heliconia**		
66	Granada (Meta)	Pseudostem	113	Palmira (Valle del Cauca)	Pseudostem
67	Fuente de Oro (Meta)	Pseudostem	114	Palmira (Valle del Cauca)	Rhizome
71	Urabá (Antioquia)	Rhizome	115	Palmira (Valle del Cauca)	Rhizome
72	Urabá (Antioquia)	Pseudostem			

The amplified products were separated by electrophoresis in 2% agarose gels with 0.5X TBE buffer, dyed with 0.001% ethidium bromide, run for 2½ h at 140 volts, and visualized under ultraviolet light in a Stratagene Eagle Eye® II. The band patterns of each primer were compared according to the presence or absence of a fragment; same-sized fragments were determined as identical.

To estimate the genetic relationships among the isolates, a dendrogram was generated from data on the 59 strains, using the CGA primer. Each fragment generated with the RAM technique was analyzed as an independent character. The same-sized DNA fragments were assumed to represent the same genetic locus, which was evaluated as absent or present. For each individual band, the value of 1 was assigned for presence and 0 for absence. Similarity among individuals was estimated, using the Dice coefficient of similarity. The dendrogram was generated, using the unweighted pair group method with arithmetic mean (UPGMA) and the statistical packet NTSYS-PC, version 2.02.

Results and Discussion

Of the seven primers evaluated, we standardized the CGA primer with 1.5 mM $MgCl_2$, managing to obtain patterns of reproducible bands for *R. solanacearum* and observing polymorphisms among the strains from different sites.

To read the bands, a range between 201 and 1327 bp was selected, observing characteristic bands for the strains from different crops and departments (Figures 1.19.1 to 1.19.3).

By analysis in NTSYS, a dendrogram (Figure 1.19.4) was prepared that generated 12 clusters, having a coefficient of similarity of 0.87 and differentiating according to crop type and geographic location. These were:

- Two of the three strains isolated from plantain in Antioquia, forming a cluster and showing 83% similarity with strains isolated from banana in Antioquia.

- The strains isolated from heliconia from Rozo (Valle del Cauca) showed 85% similarity with the strains isolated from the rhizosphere and plantain tissue from Jamundí (Valle del Cauca).

- The strains isolated from plantain in different municipalities of Quindío showed close similarity (82% to 100%) with strains from different municipalities of Meta.

- The Meta cluster showed moderate similarity (60% to 80%) with strains from plantain in Antioquia, indicating that no apparent separation of plantain strains exists, even though some clustered independently of others.

- Highest variation was observed between the strains from banana in Antioquia and those from banana in Magdalena, showing a 25% similarity and thus indicating that, between these two departments, strains possibly present the greatest genetic variation. To confirm this finding, however, a larger number of samples must be collected.

Conclusions:

The RAM technique enabled us to make an intraspecific and interspecific analysis of *R. solanacearum*, causal agent of bacterial wilt of Musaceae and of other plant species.

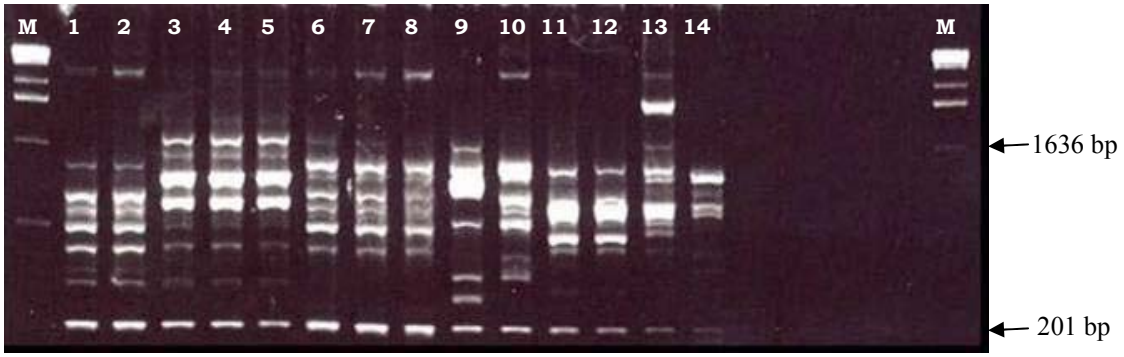

Figure 1.19.1 RAM patterns obtained with the CGA primer for *Ralstonia solanacearum* strains isolated from different plant species. M = 1-kb marker; lanes 1 and 2 = strains from banana, Antioquia, Colombia; lanes 3–5 = strains isolated from banana, Magdalena, Colombia; lanes 6–8 = strains isolated from heliconias, Valle del Cauca, Colombia; lane 9 = strain from eggplant, Kenya; lane 10 = strain from tobacco, Japan; lanes 11 and 12 = strains from tobacco, Santander, Colombia; lane 13 = strain from tobacco, Florida, USA.

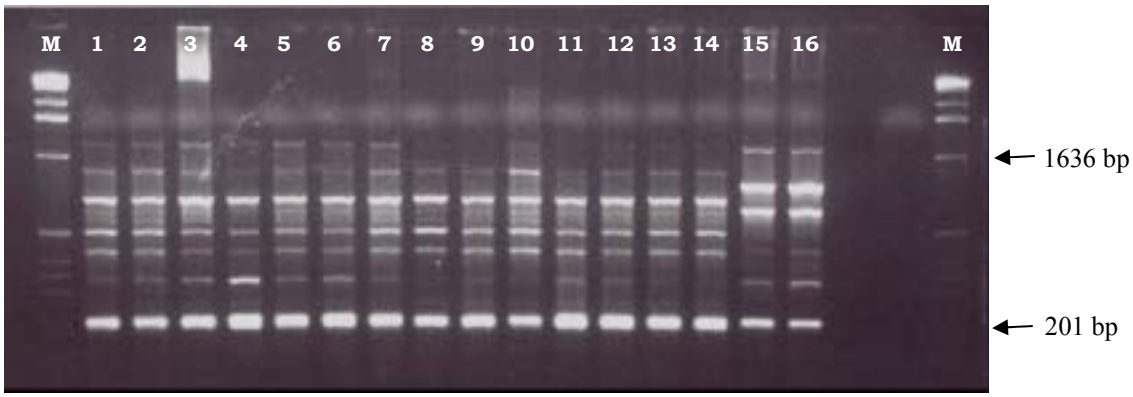

Figure 1.19.2. RAM patterns obtained with the CGA primer for *Ralstonia solanacearum* strains isolated from two different sites in Colombia. M = 1-kb marker; lanes 1–14 = strains isolated from plantain, Quindío; lanes 15 and 16 = strains isolated from banana, Magdalena.

With the CGA primer, we obtained polymorphic band patterns that were reproducible for the selected strains. This primer also showed clear discrimination between strains according to crop type, based on the absence or presence of specific bands.

Ralstonia solanacearum strains from plantain tissue and soil from plantain crops in different parts of Colombia presented a similarity that ranged from 82% to 100%, according to the RAM analysis, showing close taxonomic affinity.

Because the band patterns were distinguishable among strains of different origin, clusters were formed according to crop type and geographical origin for which a coefficient of similarity of 0.87 was obtained. We deduced that a high level of variation does not exist among strains from plantain crops, heliconias of Valle del Cauca, and banana of Antioquia. However, the last mentioned showed less than 60% similarity with strains from banana in Magdalena, eggplant (race 3), and tobacco (race 1). Genetic variation increased among strains from different races.

The microsatellite marker CGA separated the plantain strains from strains from banana, heliconia, and the soil. This finding contrasts with the results on pathogenicity for which the strains show no differences among sites, hosts, and/or tissue types. In addition, geographical differentiation was shown for strains from Antioquia.

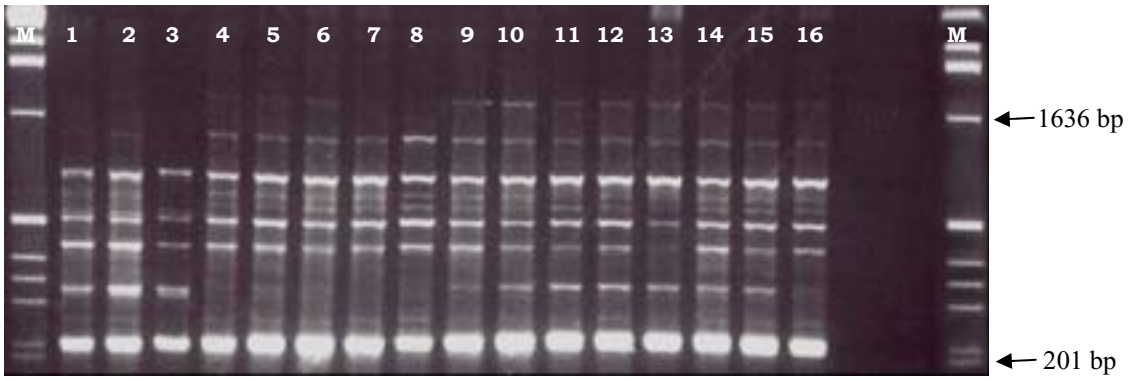

Figure 1.193. RAM patterns obtained with the CGA primer for *Ralstonia solanacearum* strains isolated from plantain in three different sites in Colombia. M = 1-kb marker; lanes 1–3 = strains from Antioquia; lane 4 = strain from Caquetá; lanes 5–16 = strains from Meta.

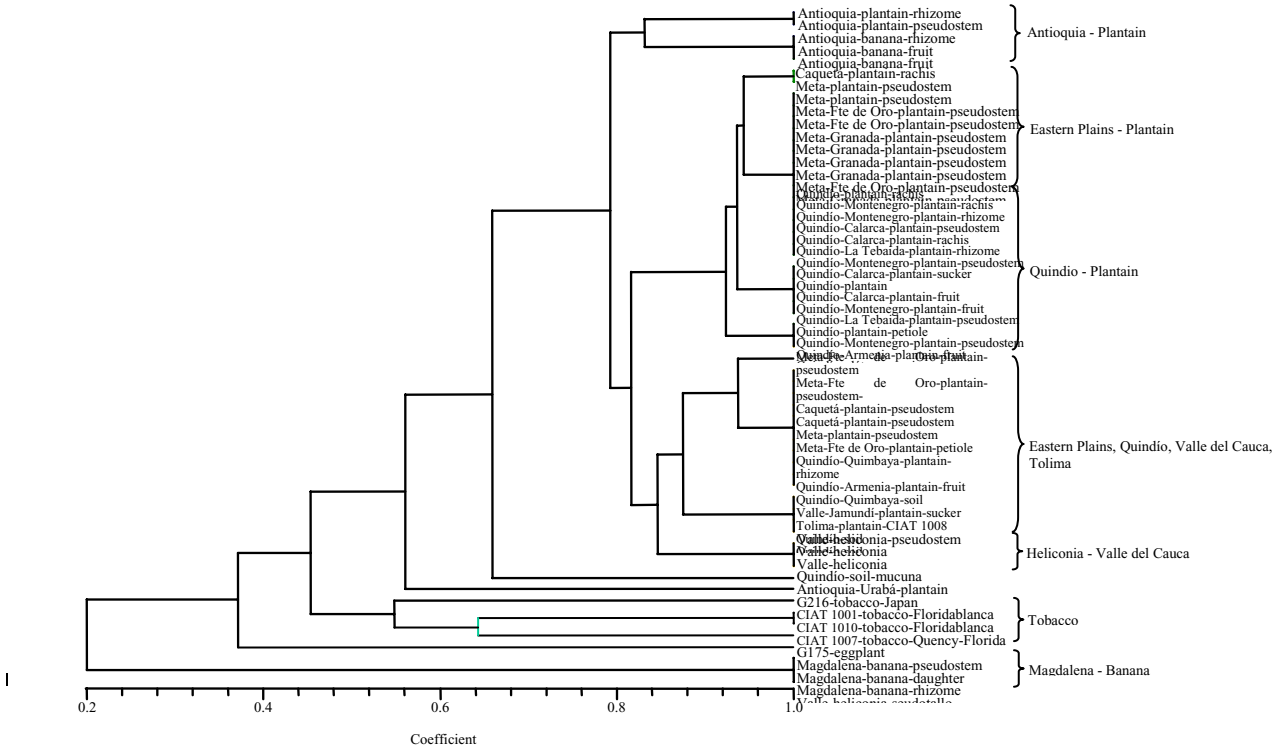

Figure 1.194. Dendrogram of similarity based on the Dice coefficient for 59 strains of *Ralstonia solanacearum*, with the RAM primer CGA.

Activity 1.20. DNA sequence analysis of the ITS region of a phytoplasma obtained from coffee: a collaborative effort between CIAT and CENICAFE

Contributors: E. Álvarez, J. F. Mejía, and C. Gálvis

Highlight:

- We confirm the association of a pathogenic agent to the coffee crispiness or *crespera*, this being the first report of a phytoplasma-caused disease in the *Coffea* genus. As a new member of the 16SrIII group, we propose the name coffee crispiness disease or CCD phytoplasma.

Rationale

Coffee crispiness is a slow-spreading disease that has been reported in Colombian coffee plantations since 1940. It is endemic to limited areas scattered throughout the country. It affects the physiological development of the aerial parts of the plant, especially the leaves and floral buds, causing leaf proliferation and phyllody, and also altering the fruits, increasing the percentages of monospermic berries. The curled leaves and massive vegetative growth that results in the branches gives the local name *crespera* to the disease.

Because no etiology has been clearly associated with coffee crispiness, control measures have been ineffective. Throughout the years, crispiness has been attributed to divergent causes, varying from viral problems, microelement deficiencies, mineral malnutrition (Drosdoff, 1956, Agricultura Tropical 12: 103 – 105), and physical and chemical soil limitations (Valencia, 1993, Fitopatología Colombiana 17:39-45; Carrillo, 1984, Federación Nacional de Cafeteros de Colombia, Informe Trimestral: 20-22). Farmers have also implicated herbicide toxicity. More recently, comparative symptomatology suggested the association of a phytoplasma with *crespera* (Moreira *et al*., 1997, Simposio Latinoamericano de Caficultura.18: 409). No evidence, however, has been provided to support any of these hypotheses.

The present work was aimed to confirme the association of a phytoplasma with coffee crispiness disease in Colombia, and to establish its phylogenetic relationship based on the molecular characterization of the 16S ribosomal DNA. We confirm a phytoplasma as the causing agent of the coffee crispiness disease. This is a necessary step in the process of understanding of the disease epidemiology, vector identification, improved diagnostic methods, and design of strategies to reduce the effect and dissemination of coffee crispiness among plantations. This is also the first report of a phytoplasma causing disease in the *Coffea* genus.

Materials and Methods

Sources of phytoplasmas: Symptomatic branches from *Coffea arabica* L. were collected from the Santa Rosa de Cabal Region (Risaralda, Colombia), in 5-year-old coffee plantations that had been regenerated from stumps. The negative control always used was *C. arabica* var. Caturra, obtained from seeds of healthy plants.

DNA extraction: DNA was totally extracted from leaves according to the procedure described by Bernatzky and Tanksley (1986, Genetics 112:887–898) but with minor modifications. Briefly, 100 mg of leaves, ground in liquid nitrogen, were immersed in 500 µL of extraction buffer (200 mM Tris-HCl, pH 8.0; 70 mM EDTA; 2 M NaCl; 20 mM sodium metabisulfate; 2% CTAB; and 0.2% ß-mercaptoethanol to prevent phenolic oxidation) and incubated in a water bath at 65 ºC for 1 h. Next, one volume of phenol was added and mixed by inversion for 10 min. It was then centrifuged at 13,000 rpm for 5 min, and the supernatant removed to another tube. An extraction with 500 εL of chloroform followed, and after

centrifuging at 6000 rpm, the supernatant was removed to a new tube. DNA was precipitated by adding 250 εL of 5 M ammonium acetate and 750 εL of isopropanol, and spun for 25 min at 14,000 rpm. After rinsing with 1 mL of 70% ethanol and a final centrifuging at 12,000 rpm for 2 min, the supernatant was discarded and, once dry, the pellet was resuspended in 50 εL of TE buffer (10 mM Tris-HCl, pH 8.0; and 0.1 mM EDTA) and treated with 5 εg of SIGMA RNAase per tube.

PCR amplification: Three primer pairs from the 16S rDNA were synthesized by Operon Technologies (Alameda, CA):

P1 (5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3') and
P7 (5'-CGT CCT TCA TCG GCT CTT-3')

R16F2 (5'-ACG ACT GCT AAG ACT GG-3') and
R16R2 (5'-TGA CGG GCG GTG TGT ACA AAC CCC G-3'); and

FU5 (5'- CGG CAA TGG AGG AAA CT-3')
rU3 (5'-TTC AGC TAC TCT TTG TAA CA-3').

A nested-PCR reaction was performed as follows: a first amplification was carried out, using Promega reagents for a Master Mix with 0.1% PCR buffer, 25 mM $MgCl_2$; 2 mM dNTPs; and 0.1 U/εL of *Taq* polymerase, in addition to 0.2 μM of primers P1 and P7; 5 εL of 20 ng/εL coffee DNA; and distilled water to complete a volume of 25 εL. An amplification program was run on an MJ Research thermal cycler, consisting of an initial denaturation at 94 ºC for 2 min, and 35 cycles of 94 ºC for 1 min, 55 ºC for 2 min, and 72 ºC for 3 min. A final extension cycle was performed at 72 ºC for 10 min.

A second amplification was accomplished with 1 εL of a 1/30 dilution in sterilized distilled water, in a similar Master Mix, containing either the primer pair R16F2/R16R2 or FU5/rU3 and running the same thermal cycler program, except that the annealing temperature was changed to 50 ºC. PCR amplification products were separated in 1% agarose gels in TBE buffer at 60 V, stained with ethidium bromide, and recorded under UV light with a digital camera.

Molecular detection in grafted coffee plants: Leaf tissue was obtained from five plants grafted with diseased branches and from six 3-year-old plants from a commercial plot in the Santa Rosa de Cabal Region. They were then compared with healthy leaf tissue by amplifying the 16S rDNA region of the phytoplasma. Primer pairs P1/P7 and FU5/rU3 were used for the nested-PCR reaction, using coffee genomic DNA.

Cloning the PCR products and DNA sequencing: Amplified fragments in PCR by FU5/rU3 were cloned in *Escherichia coli* JM109, using the pGEM®-T Easy Vector System (Promega) according to manufacturer's instructions. Transformants were selected on blue/white color screening by plating on LB/ampicillin/IPTG/X-gal media. Positive inserts were observed by plasmid restriction with *Eco*RI and electrophoresis in 1.5% agarose gel.

Different-sized fragments were selected for sequencing by automated DNA sequencing at the Cornell Biotechnology Resource Center (Ithaca, NY). Plasmid sequences were removed from each sequence and high-quality sequence data collected. The sequence results were assembled and analyzed, using BLAST®n at NCBI (http://www.ncbi.nlm.nih.gov). The nucleotide sequences determined in this study were deposited in the GenBank data library (NCBI, MD).

Phylogenetic análisis: 16S rRNA gene sequences (941 pb) from coffee phytoplasma and 42 other strains

(representing 12 16S rRNA phytoplasma groups) were obtained from the GenBank, and a primary alignment performed with ClustalX (Thompson *et al*., 1997, Nucl Acids Res 24:4876–4882), with the flanking non-consensus regions removed by editing with BioEdit (Hall, 1999, Nucl Acids Symp Ser 41:95–98). A phylogenetic tree was constructed by the neighbor-joining method of the ClustalX program. The tree was viewed by using TreeView ([Page 1996, Computer Appl Biosci 12:357–358.])

Results and Discussion

Molecular detection in coffee plants: Clear amplifications of the 941-bp band were obtained from infected field plants. Samples from grafted tissue produced weaker amplifications, but still detectable. Negative controls and healthy tissues resulted in no amplifications (Figure 1.20.1). By nested PCR, a 941-bp fragment was amplified from DNA of infected tissues (GenBank Accession Number AY525125). When a common region for the 16S rDNA of each of the 12 phytoplasma groups was aligned, the one amplified from coffee clustered with the 16SrIII group (Figure 1.20.2), described as the X-disease group by Lee *et al.*(1994, Phytopathology 84:559-66).

Further analysis with the 42 sequences described as belonging to the 16SrIII family or as having close similarity, produced a radial tree for the family, with four main branches. The coffee crispiness phytoplasma was one of the most distant sequences of the whole group, with the phytoplasma causing *machorreo* in *lulo* (or naranjilla; *Solanum quitoense*: Asteridae: Solanaceae) being the closest. It was in a cluster that also contained the phytoplasmas causing chayote witches' broom (in *Sechium edule* (Jacq.) Sw., Dilleniidae: Cucurbitaceae) and garlic wilt (in *Allium sativum* L., Lilidae: Liliaceae) (Figure 1.20.3).

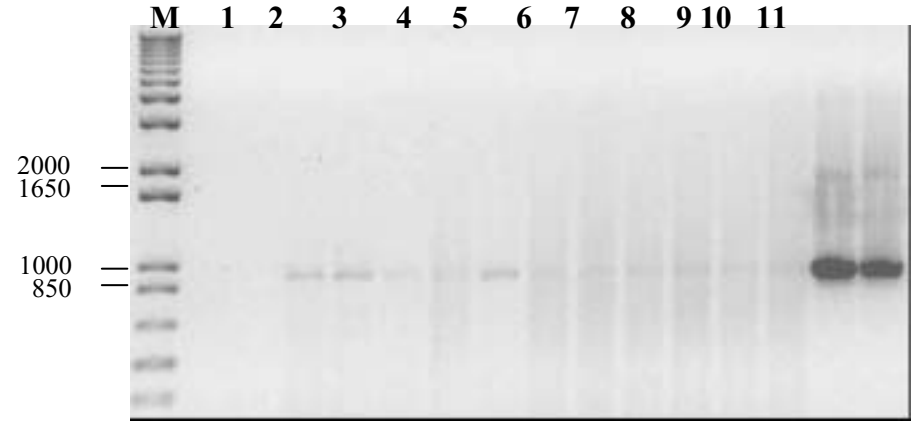

Figure 1.20.1. Agarose gel separation of PCR products from nested amplification of a 941-bp band corresponding to the 16S rDNA. M = molecular weight marker; lane 1 = PCR control; lane 2 = healthy coffee tissue; lanes 3 to 7 = field plant tissue exhibiting crispiness; lanes 7 to 13 = rootstock from grafted tissue, with symptoms; lanes 14 and 15 = cloned and sequenced crispiness phytoplasma rDNA.

The possible taxonomic association between the *machorreo* and coffee crispiness phytoplasmas suggests the presence of derivatives from a common ancestor in South America. However, while the Andes are considered as the geographic origin of Solanaceae, coffee was introduced only within the last 2 centuries. This suggests the hypothesis that coffee became an alternative host for a phytoplasma that more efficiently affects other plant species. In fact, the limited distribution, and moderate dispersion and virulence of crispiness agree with pathogen behavior and may provide alternatives for disease control. Moreover, the

same clade carries the China-tree phytoplasma, reported in Bolivia, the chayote witches' broom from Brazil, and the garlic decline phytoplasma from Argentina (Galdeano *et al*., 2004, Journal of Phytopathology, 152: 174-181).

We confirm the association of a pathogenic agent to the coffee crispiness or *crespera*, this being the first report of a phytoplasma-caused disease in the *Coffea* genus. As a new member of the 16SrIII group, we propose the name coffee crispiness disease or CCD phytoplasma.

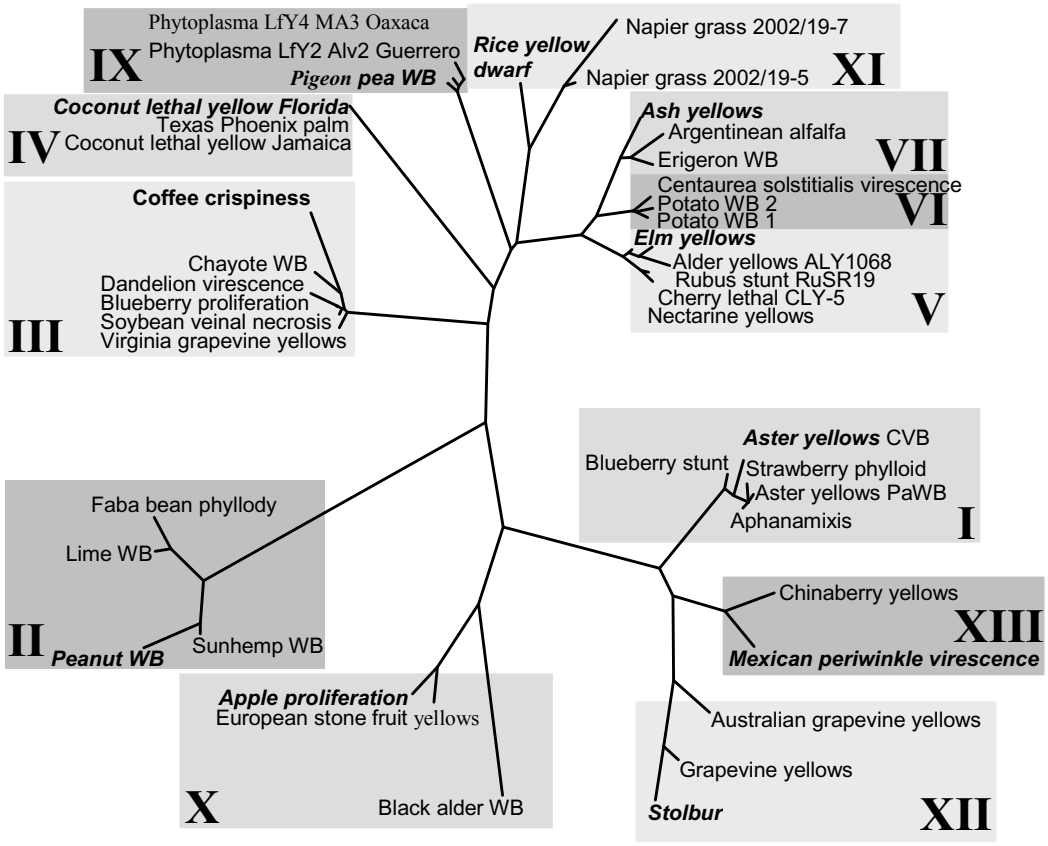

Figure 1.20.2. Dendrogram based on the 16S rDNA sequences of representative phytoplasmas retrieved from GenBank. The coffee crispiness phytoplasma groups with the 16SrIII (X-disease) group.

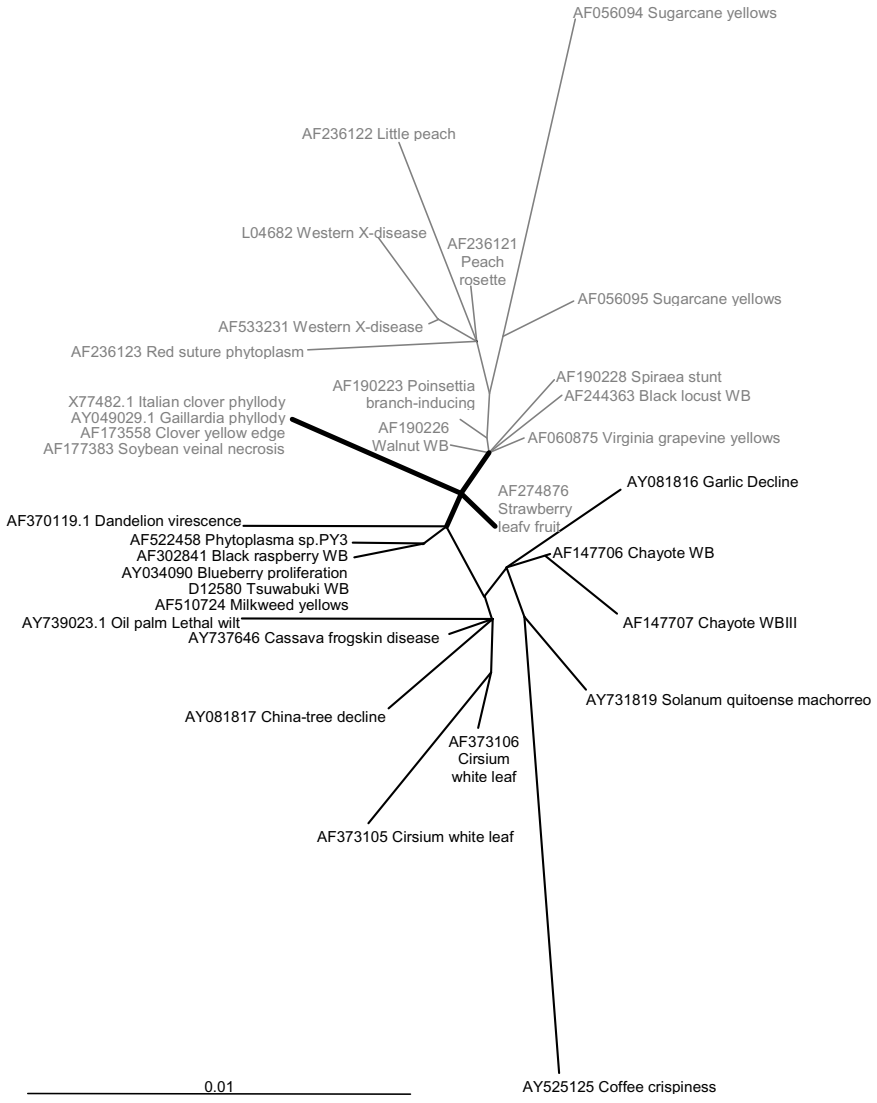

Figure 1.20.3. Detailed organization of the 16SrIII (X-disease) phytoplasma group. The closest relative to the coffee crispiness phytoplasma is the Solanum quitoense *machorreo* phytoplasma, also isolated in Colombia. (WB = witches' broom; thick black lines = major branches)

Activity 1.21. Identifying and Characterizing Strains of *Ralstonia solanacearum* Race 2, Causal Agent of *Moko* of Plantain in Colombia

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Highlight:

∉ A BIO–PCR technique was developed for detecting *R. solanacearum*. This technique had increased sensitivity, and detected only live cells of the pathogen in soil and plant tissue.

Rationale

Moko, *maduraviche*, or *ereke* is a bacterial wilt of plantain and banana caused by *Ralstonia solanacearum* E.F. Smith race 2 (Yabucchi *et al*., 1995, cited by Ito *et al*., 1998, In: Journal of Phytopathology 146: 379 – 384). It is the most important bacterial disease of these crops in Colombia, affecting possibly 125,000 families who depend directly on them for their livelihoods. Currently, despite dissemination of preventive measures and disease management, the disease is spreading, to the point where 95% of plantain fields have at the least one plant with *moko* (personal communication, Galindo 2004, ICA, Bogotá).

The BIO-PCR technique developed by Schaad *et al.* (1995, Phytopathology 85:243-248) improves efficiency in detecting viable cells of this pathogen, especially in soil. This technique consists of isolating colonies in semi-selective medium, South Africa (SMSA). It shows higher sensitivity and specificity than does triphenyltetrazolium chloride (TTC) (Kelman, 1954, Phytopathology 44: 693-695) with later amplification by polymerase chain reaction (PCR). *Ralstonia solanacearum* is variable in the range of hosts it attacks, geographical distribution, pathogenicity, epidemiological relationships, and physiological properties. Hence, in the last three decades, races and biovars have been used informally to classify the pathogen at the infra-subspecific level (not governed by the *International Code of Nomenclature of Bacteria*).

The main objective of our studies is to isolate *R. solanacearum* from soil and from infected plant tissue, using BIO-PCR, culture medium SMSA, specific primer OLI 1, and nonspecific primer Y2 in order to improve efficiency in detecting the pathogen. We also characterized the pathogen with the objective to select plantain cultivars with more durable resistance to *moko* disease and to develop better control practices.

Materials and Methods

Sample sources: We processed 134 samples of infected plant tissue from pseudostems, rachis, fruits, and rhizomes of selected plantain, banana, and heliconia plants that had presented typical symptoms of the disease. We also processed soil samples from farms affected by moko and located in the production areas of the Departments of Valle del Cauca, Quindío, Antioquia, Caquetá, Meta, and Magdalena in Colombia (Table 1.21.1).

Processing plant-tissue simples: The bacterium was extracted from selected infected tissue fragments, which had been washed, disinfected, and macerated in a mortar containing a buffer solution of 10 mM Tris-HCl and 1 mM EDTA at a pH = 7.6. This suspension was cultured, using a sterilized micro-spade, in petri dishes containing the semi-selective culture medium, South Africa (SMSA), a modification of the medium triphenyltetrazonium chloride (TTC). The SMSA medium contained 10 g/L peptone, 5 mL/L glycerol, 1 g/L casamino acids, 18 g/L agar, antibiotics (100 mg/L, i.e., 600,000 U polymyxin η sulfate; 25 mg/L bacitracin; 0.5 mg/L, i.e., 82.5 U penicillin, and 5 mg/L chloramphenicol), 50 mg/L of 2,3,5-TTC, and 5 mg/L crystal violet (Denny and Hayward, 2001, Laboratory guide for identification of plant pathogenic bacteria (APS): 151-173 ; Englebrecht 1994). The dishes cultured with the suspension were incubated at a temperature of 28 ºC for 3 to 5 days, depending on when the colonies appeared.

Processing soil simples: With soil taken from around plantain plants infected by the bacterium, suspensions were prepared by adding 3.3 g soil to 30 mL TE buffer at pH = 7.6. Serial dilutions were carried out in TE buffer. We then took 100 µL of each of the dilutions 10^{-1}, 10^{-2}, and 10^{-3}, culturing into petri dishes containing SMSA medium. The dishes were incubated at 28 ºC for 3 to 5 days, depending on when the colonies first appeared.

Isolating and testing the bacterium: From the samples, we selected bacterial colonies that showed similar

growth patterns to those of the *R. solanacearum* control strain CIAT 1008. The control strain came from Ibagué (Tolima, Colombia) and was held at the strain bank in the Cassava Pathology Laboratory, CIAT.

Purified bacterial colonies belonging to the Gram-negative group of bacteria were selected, using the KOH (3%) test. A drop of this reagent was placed on a glass slide and a colony from a pure and metabolically active culture with a 24-h growth then dissolved in it. The reaction was considered positive when a mucous thread could be seen on lifting the micro-spade from the bacterial suspension.

The oxidase test was carried out by placing two drops of a 1% aqueous solution of dichlorohydrate of tetra-methyl-p-phenylenediamine on a strip of filter paper, which was then rubbed over a colony. The reaction was considered positive when the solution in the paper turned from colorless to dark purple within the next 30 to 60 s. The strain CIAT 1008 was included as check for both tests.

Extracting DNA and conducting PCR: Genomic DNA was extracted from pure colonies of selected strains with a 36-h growth in nutritive agar (Seal *et al*., 1999, Plant Pathology 48: 115 – 120). Each colony was suspended in vials containing 100 σL of sterilized distilled water, and heated in a bain-marie at 96 ºC for 5 min. The vials were then centrifuged at 12,000 rpm for 2 min and 2.5 σL of the supernatant taken as DNA mold for the polymerase chain reaction (PCR).

The volume of the cocktail for amplification was 9.98 µL, which contained a 1.25X buffer for *Taq* polymerase; 0.012 mM of each dNTP; 1.87 mM $MgCl_2$; 0.25 U *Taq* polymerase; and 0.16 σM of each of the primers OLI 1 (5'-GGGGGTAGCTTGCTACCTGCC-3') and Y2 (5'-CCCACTGCTGCCTCCCGTAGGAGT-3'), (Martins, 2000, Thesis: Polymerase Chain Reaction in the diagnosis of Bacterial Wilt caused by *Ralstonia solanacearum* Georg-August University, Gottingen, Germany, p127; Seal *et al*., 1999, Plant Pathology 48: 115 – 120).

Determining biovars: The reaction of each of 72 strains (8 from soil and 64 from plant tissue) to sugars and alcohols indicated that all the strains characterized belonged to biovar 1. They had not used any of the three sugars, nor oxidized the three hexose alcohols used in the biochemical tests.

Pathogenicity test and confirmation of race 2: The reaction of hypersensitivity obtained in tobacco leaves 48 h after inoculation indicated that 63 of the 72 strains caused a typical hypersensitivity reaction in tobacco leaves (Figure 1.21.2). The remaining 9 strains induced yellowing, an atypical reaction of hypersensitivity for this race. Eight of these strains came from the Colombian Atlantic Coast (Urabá).

Seventy-one strains were pathogenic when inoculated into plantain plants, confirming that they belonged to race 2. Only one strain, which came from Urabá, was not pathogenic (Figure 1.21.3). The separation-of-means test, estimated through MSD (ζ = 5%), led to grouping the strains in three categories according to their pathogenicity (AUDPC). The highly pathogenic strains had AUDPC values between 45.13 and 73.38; the strains with moderate pathogenicity showed values between 18.00 and 43.13; and the strains with low pathogenicity had values between 0 and 15.75 (Table 1.21.2)

The DNA was amplified in an MJ Research PTC-100 thermal cycler, using the following program: initial denaturation for 2 min at 96 ∀C; 50 denaturation cycles, e ach for 20 s at 94 ∀C; annealing for 20 s at 62 ∀C; extension for 30 s at 72 ∀C; and a final extension of 5 min at 72 ∀C (Seal *et al*., 1999). The PCR products were separated in 1.5% agarose gels, dyed with 0.001% ethidium bromide, and visualized under ultraviolet light. Evaluations were based on the presence of a band, 287–288 base pairs long, from the 16S rRNA fragment generated by amplification with the specific primer OLI 1 and the nonspecific primer Y2 (Seal *et al*., 1999, Plant Pathology 48: 115 – 120).

Table 1.21.1. Samples of plant tissue used to isolate *Ralstonia solanacearum* race 2, causal agent of *moko* (bacterial wilt) of plantain, according to origin by department, crop, and source of isolate.

Sample No	Origin			Sample No	Origin		
	Department/ Locality	Crop	Source		Department/ Locality	Crop	Source
1	Quindío	Plantain	Rachis	79	Montenegro (Quindío)	Plantain	Rhizome
2	Quindío	Plantain	Petiole	80	Montenegro (Quindío)	Plantain	Pseudostem
3	Quindío	Plantain	Petiole	81	Montenegro (Quindío)	Plantain	Fruit
4	Urabá (Antioquia)	Banana	Pseudostem	83	Quindío	Plantain	Fruit
5	Urabá (Antioquia)	Banana	Rhizome	84	Quindío	Plantain	Pseudostem
6	Urabá (Antioquia)	Banana	Fruit	85	Quindío	Plantain	Sucker
7	Urabá (Antioquia)	Banana	Fruit	86	Calarcá (Quindío)	Plantain	Rachis
15	Quindío	Plantain	Soil	88	LaTebaida (Quindío)	Plantain	Rhizome
17	Jamundí (Valle)	Plantain	Soil	89	LaTebaida (Quindío)	Plantain	Pseudostem
18	Jamundí (Valle)	Plantain	Sucker	90	Montenegro (Quindío)	Plantain	Petiole
32	Caquetá	Plantain	Pseudostem	91	Montenegro (Quindío)	Plantain	Rachis
33	Caquetá	Plantain	Pseudostem	92	Quimbaya (Quindío)	Plantain	Petiole
34	Caquetá	Plantain	Rachis	94	Quimbaya (Quindío)	Plantain	Rachis
38	Quindío	Plantain	Soil	95	Quimbaya (Quindío)	Plantain	Rhizome
39	Quindío	Plantain	Soil	96	Quimbaya (Quindío)	Plantain	Pseudostem
40	Quimbaya (Quindío)	Plantain	Soil	97	Quimbaya (Quindío)	Plantain	Rhizome
41	Quimbaya (Quindío)	Plantain	Soil	98	Quimbaya (Quindío)	Plantain	Rachis
42	Fuente de Oro (Meta)	Plantain	Pseudostem	99	Quimbaya (Quindío)	Plantain	Pseudostem
43	Fuente de Oro (Meta)	Plantain	Pseudostem	100	Armenia (Quindío)	Plantain	Sucker
48	Armenia (Quindío)	Plantain	Fruit	101	Armenia (Quindío)	Plantain	Pseudostem
54	Fuente de Oro (Meta)	Plantain	Pseudostem	102	Quimbaya (Quindío)	Plantain	Petiole
55	Fuente de oro (Meta)	Plantain	Pseudostem	104	Armenia (Quindío)	Plantain	Fruit
57	Fuente de oro (Meta)	Plantain	Pseudostem	106	Armenia (Quindío)	Plantain	Pseudostem
58	Fuente de oro (Meta)	Plantain	Pseudostem	107	Armenia (Quindío)	Plantain	Fruit
59	Fuente de oro (Meta)	Plantain	Pseudostem	109	Armenia (Quindío)	Plantain	Petiole
60	Fuente de oro (Meta)	Plantain	Pseudostem	110	Magdalena	Banana	Pseudostem
63	Granada (Meta)	Plantain	Pseudostem	111	Magdalena	Banana	Rhizome
64	Granada (Meta)	Plantain	Pseudostem	112	Magdalena	Banana	Sucker
65	Granada (Meta)	Plantain	Pseudostem	113	Palmira (Valle)	Heliconia	Pseudostem
66	Granada (Meta)	Plantain	Pseudostem	114	Palmira (Valle)	Heliconia	Rhizome

Table 1.21.1, (cont'd)

Sample No	Origin			Sample No	Origin		
	Department/ Locality	Crop	Source		Department/ Locality	Crop	Source
69	Granada (Meta)	Plantain	Pseudostem	160	Quindío	Plantain	Soil
70	Granada (Meta)	Plantain	Pseudostem	161	Quindío	Plantain	Soil
71	Urabá (Antioquia)	Plantain	Rhizome	588	Fuente de Oro (Meta)	Plantain	Petiole
72	Urabá (Antioquia)	Plantain	Pseudostem	CIAT 1008[4]	Ibagué (Tolima)	Plantain	Sin Inf.
73	Urabá (Antioquia)	Plantain	Pseudostem				
76	Montenegro (Quindío)	Plantain	Pseudostem				
78	Montenegro (Quindío)	Plantain	Rachis				

Biovar determination: Ralstonia solanacearum strains can be classified into different biovars according to Hayward (1964, Annual review of phytophatology 29: 64-87) by their production of acids from the disaccharides cellobiose, lactose, and maltose and by their oxidation of the hexose alcohols sorbitol, dulcitol, and mannitol in base medium (Denny and Hayward, 2001, Laboratory guide for identification of plant pathogenic bacteria., (APS), 151-173).

The base medium contained (per liter) 1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4.7H_2O$, 1.0 g Bacto™ Peptone, 3.0 g agar, and 80.0 mg bromothymol blue with a pH between 7.0 and 7.1, becoming green olive. The medium was then sterilized by autoclaving at 121 ºC, with a 20-lb pressure, for 20 to 30 min (Denny and Hayward, 2001, Laboratory guide for identification of plant pathogenic bacteria, (APS), 151-173).

We also prepared 10% aqueous solutions of each of the test carbohydrates and sterilized them by filtration, using Millipore® filters with a pore size of 0.22 µm. These solutions of carbohydrates were added to the sterilized base medium when the temperature was between 55 and 60 ºC, obtaining a final concentration of 1%. After mixing the base medium with each sugar, about 5 mL of the liquid medium placed within tubes containing sterilized cultures (Denny and Hayward, 2001, Laboratory guide for identification of plant pathogenic bacteria, (APS), 51-173).

The medium was inoculated with the bacterium by means of puncturing as deep as three quarters of the medium, using colonies with a 24-h growth in nutritive agar. Reactions were assessed after 1, 3, 7, 14, and 28 days of incubation at 28 ºC. The color changed from green olive to yellow as acids were produced from the disaccharides and the hexose alcohols were oxidized (Denny and Hayward, 2001, Laboratory guide for identification of plant pathogenic bacteria, (APS), 151-173).

Testing for pathogenicity in plantain: The strains identified by PCR as being *R. solanacearum* were inoculated into plantain plants of 'Africa' (*Musa* cv. AAB) derived from in vitro meristem culture. At 15 days old, the plantlets were transplanted to plastic bags carrying 1 kg of sterilized sand and soil mixed in a 3:2 ratio. For the next 15 days, the plants were continuously humidified to guarantee their optimal development.

The plants were not watered for 24 h before inoculation. For each strain of *R. solanacearum*, four plantain plants were inoculated at about 6 weeks old by injection of the pseudostem, using sterilized 1-mL syringes with needle size 27G × 1/2". Injection was to the center of the pseudostem at 2 cm above the soil surface.

The substance injected comprised 0.5 mL of bacterial suspension made with pure bacterial cultures with a 24-h growth in nutritive agar that were suspended in sterilized deionized water. The concentration of the

suspension was determined by absorbance readings in a spectrophotometer and adjusted to 0.1 with a wavelength of 600 nm. This corresponded to about 1×10^{-8} cfu · mL^{-1} (He *et al*., 1983, Plant Disease 67: 1357-1361).

As positive check, the pathogenic strain *R. solanacearum* CIAT 1008 was used. The negative check was inoculated sterilized water. The inoculated plants remained under controlled conditions of temperature between 24 and 29 ºC, about 13 h of light, and relative humidity from 80% to 91% for the first 4 days, with humidification being later reduced to 1 h per day.

Severity of symptoms were evaluated in terms of wilt, using a visual scale of 1 to 5, where 1 referred to a plant with 1 wilted leaf and 5 to a plant with five wilted leaves. Daily evaluations were made over 18 days, starting from the fourth day after inoculation, for symptoms such as flaccid leaves, wilting, and stunting. With this information, the area under the disease progress curve (AUDPC) was calculated. In preliminary research (unpublished data), we had found that, from day 5, plants can show disease symptoms such as flaccid and/or wilting leaves.

Hypersensitivity test: The capacity of the strains to induce a hypersensitivity reaction was tested in leaves of tobacco (*Nicotiana tabacum*). From pure cultures, a suspension was prepared in sterilized deionized water, using colonies with 24 h of incubation in nutritive agar and an absorbance of 0.1 with a 600-nm wavelength, thus corresponding to a concentration of about 1×10^{8} cfu · mL^{-1} (He *et al.* ,1983, Plant Disease 67: 1357-1361)

In this test, 8-week-old tobacco plants were used. These were inoculated by infiltration of the bacterial suspension, injecting with a 1-mL syringe into the veins on the lower side of leaves, permitting distribution of the suspension in the palisade layer of the parenchyma. Two leaves per plant and two plants per strain were inoculated.

The reaction was evaluated, beginning 16 h after inoculation, for symptoms corresponding to hypersensitivity to race 2 of *R. solanacearum*. These are chlorosis in infected cells of the parenchyma; wet tissue limited by a defined margin of uninoculated tissue; the area of infiltrated leaf becoming, between 36 and 60 h later, necrotic and dry from water loss; and, finally, the affected area becoming thin, white, and translucent (Lozano and Sequeiro, 1970, Phytopathology 60: 833-838).

Data analysis: An analysis of variance was carried out for the variable AUDPC. A test for the separation of means by minimum significant difference (MSD; $\zeta = 5\%$) was also conducted to separate the strains into groups according to their levels of pathogenicity (Table 1.21.2).

Results and Discussion

Isolating Ralstonia solanacearum: Samples from six regions in Colombia were taken from soil in plantain crops affected by *moko* and from plant tissues of infected plantain, banana, and heliconias. From these samples, 189 strains of the bacterium were initially selected for their growth in SMSA medium. This growth was similar to that of the *R. solanacearum* control strain CIAT 1008 when observed 48 h after incubation at 28 ∀C. This medium reduced the growth of saprophytic bacteria.

Analysis through the polymerase chain reaction (PCR): In a 1.5% agarose gel, a band with a molecular weight of 288 bp was detected. For 106 of the 189 strains obtained, the fragment was located in gene 16S rRNA, which enabled us to identify them as *R. solanacearum* (Figure 1.21.1).

Table 1.21.2. Origin and pathogenicity of 72 strains of *Ralstonia solanacearum* race 2, causal agent of *moko* (bacterial wilt), isolated from banana, plantain, and heliconias.

Strain No	Origin		Path'y			Hypers.[4]	Strain no.	Origin		Path'y			Hypers.[4]
	Dep't or locality	Crop	Source[1]	AUDPC[2]	Group[3]			Dep't or locality	Crop	Source[1]	AUDPC[2]	Group[3]	
1	Quindío	Pl.	R.	18,00	2	+	79	Montenegro (Quindío)	Pl.	Rh.	66,88	1	+
2	Quindío	Pl.	Pe.	69,38	1	+	80	Montenegro (Quindío)	Pl.	Ps.	67,88	1	+
3	Quindío	Pl.	Pe.	49,00	1	+	81	Montenegro (Quindío)	Pl.	F.	10,88	3	+
4	Urabá (Antioquia)	B.	Ps.	43,13	2	-	83	Quindío	Pl.	F.	55,00	1	+
5	Urabá (Antioquia)	B.	Rh	38,75	2	-	84	Quindío	Pl.	Ps.	61,00	1	+
6	Urabá (Antioquia)	B.	F.	31,83	2	-	85	Quindío	Pl.	Su.	68,38	1	+
7	Urabá (Antioquia)	B.	F.	62,17	1	-	86	Calarcá (Quindío)	Pl.	R.	59,75	1	+
15	Quindío	Pl.	S.	37,63	2	+	88	La Tebaida (Quindío)	Pl.	Rh.	61,75	1	+
17	Jamundí (Valle)	Pl.	S.	69,50	1	+	89	La Tebaida (Quindío)	Pl.	Ps.	60,38	1	+
18	Jamundí (Valle)	Pl.	Su.	42,50	2	+	90	Montenegro (Quindío)	Pl.	Pe.	19,25	2	-
32	Caquetá	Pl.	Ps.	33,88	2	+	91	Montenegro (Quindío)	Pl.	R.	19,38	2	+
33	Caquetá	Pl.	Ps.	40,38	2	+	92	Quimbaya (Quindío)	Pl.	Pe.	63,13	1	+
34	Caquetá	Pl.	R.	27,63	2	+	94	Quimbaya (Quindío)	Pl.	R.	47,25	1	+
38	Quindío	Pl.	S.	59,50	1	+	95	Quimbaya (Quindío)	Pl.	Rh.	65,63	1	+
39	Quindío	Pl.	S	62,00	1	+	96	Quimbaya (Quindío)	Pl.	Ps.	33,00	2	+
40	Quimbaya (Quindío)	Pl.	S.	15,75	3	+	97	Quimbaya (Quindío)	Pl.	Rh.	28,63	2	+
41	Quimbaya (Quindío)	Pl.	S.	56,25	1	+	98	Quimbaya (Quindío)	Pl.	R.	59,50	1	+
42	Fuente de Oro (Meta)	Pl.	Ps.	28,00	2	+	99	Quimbaya (Quindío)	Pl.	Ps.	40,25	2	+
43	Fuente de Oro (Meta)	Pl.	Ps.	20,75	2	+	100	Armenia (Quindío)	Pl.	Su.	71,88	1	+
48	Armenia (Quindío)	Pl.	F.	37,13	2	+	101	Armenia (Quindío)	Pl.	Ps.	58,50	1	+
54	Fuente de Oro (Meta)	Pl.	Ps.	36,25	2	+	102	Quimbaya (Quindío)	Pl.	Pe.	1,38	3	+
55	Fuente de oro (Meta)	Pl.	Ps.	55,25	1	+	104	Armenia (Quindío)	Pl.	F.	41,25	2	+
57	Fuente de oro (Meta)	Pl.	Ps.	45,63	1	+	106	Armenia (Quindío)	Pl.	Ps.	24,75	2	+
58	Fuente de oro (Meta)	Pl.	Ps.	56,63	1	+	107	Armenia (Quindío)	Pl.	F.	68,25	1	+
59	Fuente de oro (Meta)	Pl.	Ps.	0,00	3	+	109	Armenia (Quindío)	Pl.	Pe.	45,13	1	+
60	Fuente de oro (Meta)	Pl.	Ps.	47,25	1	+	110	Magdalena	B.	Ps.	63,25	1	+

Table 1.21.2 (cont'd)

Strain No	Origin		Path'y				Strain No.	Origin		Path'y			
	Dep't or locality	Crop	Source[1]	AUDPC[2]	Group[3]	Hypers.[4]		Dep't or locality	Crop	Source[1]	AUDPC[2]	Group[3]	Hypers.[4]
63	Granada (Meta)	Pl.	Ps.	50,69	1	+	111	Magdalena	B.	Rh.	34,38	2	+
64	Granada (Meta)	Pl.	Ps.	46,13	1	+	112	Magdalena	B.	Su.	29,50	2	-
65	Granada (Meta)	Pl.	Ps.	47,63	1	+	113	Palmira (Valle)	H.	Ps.	40,50	2	+
66	Granada (Meta)	Pl.	Ps.	69,75	1	+	114	Palmira (Valle)	H.	Rh.	40,38	2	+
67	Fuente de oro (Meta)	Pl.	Ps.	41,63	2	+	115	Palmira (Valle)	H.	Rh.	33,63	2	+
69	Granada (Meta)	Pl.	Ps.	27,00	2	+	160	Quindío	Pl.	S.	12,38	3	+
70	Granada (Meta)	Pl.	Ps.	5,75	3	+	161	Quindío	Pl.	S.	1,75	3	+
71	Urabá (Antioquia)	Pl.	Rh.	21,25	2	-	588	Fuente de Oro (Meta)	Pl.	Pe.	71,25	1	+
72	Urabá (Antioquia)	Pl.	Ps.	10,75	3	-	CIAT 1008[5]	Ibagué (Tolima)	Pl.	No date.	65,13	1	+
73	Urabá (Antioquia)	Pl.	Ps.	10,75	3	-							
							DMS ζ= 5%, 28,33						
76	Montenegro (Quindío)	Pl.	Ps.	61,88	1	+							
78	Montenegro (Quindío)	Pl.	R.	73,38	1	+							

1. Pl = plantain; B = banana; H = heliconia; R = rachis; Pe = petiole; Ps = pseudostem; Rh = rhizome; Su = sucker; F = fruit; S = soil.
2. AUDPC = area under the disease progress curve.
3. Group = pathogenicity group, where 1 = high pathogenicity; 2 = moderate pathogenicity; 3 = low pathogenicity.
4. Hypers. = hypersensitivity, where + = typical hypersensitive reaction; - = atypical yellowing reaction.
5. Check strain from the CIAT collection,

Of seven strains isolated from banana, five were moderately and two were highly pathogenic. Three strains from heliconias showed moderate levels. Differences in pathogenicity were found among strains isolated from soil or various plant tissues, for example, those from rhizomes and rachis were more highly pathogenic than those from other tissues. No relationship was found between pathogenicity and geographical origin (Table 1.21.2).

Conclusions

The BIO-PCR technique facilitated detection of the pathogen in soil and plants. All the *R. solanacearum* strains isolated corresponded to biovar 1.The strains showed variation in pathogenicity according to the crop and tissue from which they were isolated.

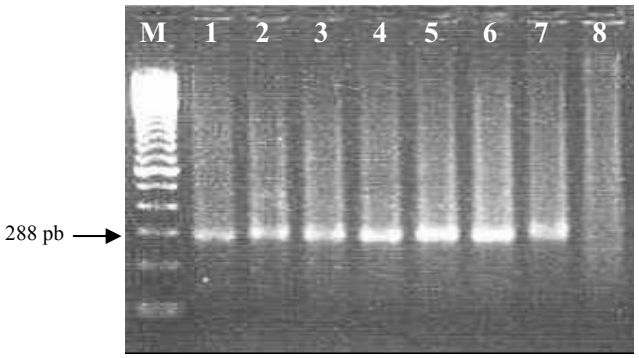

Figure 1.21.1. The figure shows the characteristic band of the bacterium *Ralstonia solanacearum*, causal agent of *moko* (bacterial wilt) in plantain. The product, measuring 288 bp, was amplified with primers OLI 1 and Y2 in the 16S rRNA region. M = 100-bp marker; lanes 1 to 3 = strains from Quindío, Colombia; lanes 4 to 6 = strains from Urabá, Colombia; lane 7 = CIAT 1008; lane 8 = negative control.

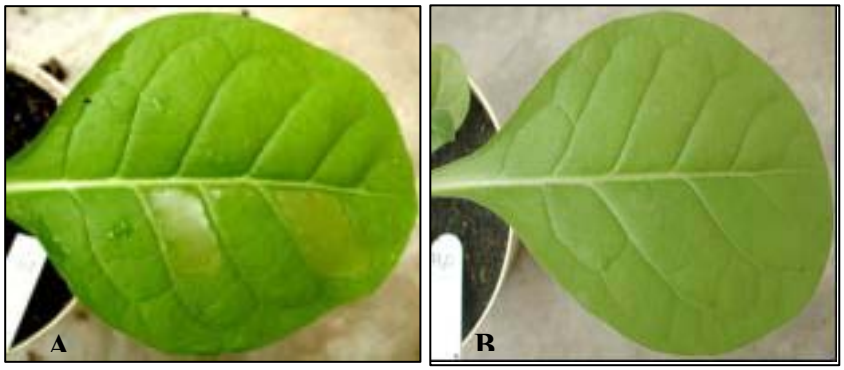

Figure 1.21.2. (A) Typical reaction of hypersensitivity in tobacco leaf, 48 h after inoculation with strain 79 of *Ralstonia solanacearum* race 2, causal agent of *moko* (bacterial wilt) in plantain. (B) Control inoculated with sterilized distilled water.

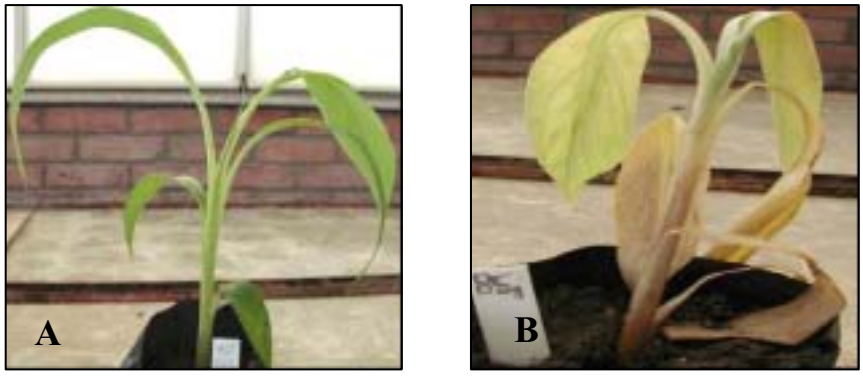

Figure 1.21.3. Plantain inoculated the center of the pseudostem at 2 cm above the soil surface, under greenhouse conditions: (A) Control whit water. (B) Wilt and yellowing of leaves caused by strain 85 of *R. solanacearum* race 2.

Activity 1.22. Molecular characterization of isolates of *Colletotrichum* spp. infecting tree tomato, mango and lemon Tahiti in Colombia.

Contributors: M. Cadavid, J. Osorio (CORPOICA) and S. Kelemu

Rationale

Colombia dedicates approximately 42,000 hectares of land to production of citrus, and 13,500 and 7,500 hectares to tree tomato and mango, respectively (Páez, 1995, ASCOLFI-Informa 21:36-39). The disease anthracnose caused by the fungal pathogen *Colletotrichum* spp. is a major production constraint resulting in losses in the range of 50-100% in various production zones. Anthracnose disease symptoms include fruit rots and blights in shoots, leaf and flowers. The disease can cause up to 50% yield loss in citrus in areas such as Valle del Cauca, Piedemonte and some areas in Magdalena (Osorio, 2000, unpublished results). In tree tomato, the disease directly affects the fruit causing total losses in areas such as Antioquia, Caldas, Risaralda, Cesar, Cundinamarca, Boyacá, Huila, Magdalena, Nariño, Tolima, Cauca andValle del Cauca, in the absence of control measures are taken, and losses between 10-25% under continuous use of fungicides. Mango anthracnose symptoms include blossom and leaf blight, fruit lesions and in severe cases tree dieback.

Effective control measure of the disease in various fruit crops is complicated by the complexity of the pathogen population structure and high variability. The high variability in morphology of *Colletotrichum* spp. in culture and the wide host range makes it difficult to use these criteria for taxonomic purposes. Molecular tools have been used for a more reliable species identification method. The objectives of this study are: 1) to characterize the pathogen population structure infecting mango, tree tomato and lemon tahiti, and 2) to use molecular approach and determine the species infecting these fruit crops. We report here very preliminary results of this work.

Materials and Methods

Fungal isolates: Thirty-five monoconidial isolates of *Colletotrichum* spp. that are maintained at the Integrated Disease and Pest Management Program of CORPOICA were used (Table 1.22.1). The isolates were obtained from naturally diseased tissues in various regions of Colombia. The isolates were grown on oatmeal agar at 28 ∀C for 5-8 days for DNA isolation. For DNA isolations, fungal cultures were grown in V-8 tomato juice broth supplemented with 10 σg/ml of streptomycin and incubated at 28∀C for 8 days in the dark and in a shaker at 130 rpm. A *C. gloeosporioides* isolate CIAT 16100 was included as a control.

DNA extraction: DNA was isolated using methods described previously (Kelemu *et al*., 1999, European Journal of Plant Pathology 105: 261-272). DNA concentration was quantified using DyNA QUANT 200⊇, aliquot at concentrations of 20ng/σl, and stored at –80 ∀C for further analysis.

Polymerase chain reaction (PCR) amplifications: For random amplified polymorphic DNAs (RAPD) analysis and primer screening, several arbitrary 10-base, oligonucleotide primers from Operon Technologies (Alameda, CA) were used for polymerase chain reaction (PCR) amplification. Amplification conditions were as described earlier (Kelemu *et al*., 1999, European Journal of Plant Pathology 105: 261-272). PCR primers for taxonomic purposes included internal transcribed spacer, ITS4 (5'-TCCTCCGCTTATTGATATGC-3'),*C.gloeosporioides* (CgInt) [5'-GGCCTCCCGCCTCCGGGCGG-3'] and *C. acutatum* (CaInt2) [5'- GGGGAAGCCTCTCGCGG-3'] To determine *C. acutatum*, the primers ITS4 and CaInt2 were used. PCR reactions were conducted in a total volume of 20 σl, containing 40 ng of DNA, 1.5 mM $MgCl_2$, 200 σM each of dNTP, 0.3 σM each of the primers, 1 unit of Taq Polimerasa Promega⊇ (Promega Corp, Madison, WI), and 1X buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1%

Triton⊇ X-100). Amplifications were carried out in a PTC-100 thermal cycler (MJ Research, Inc, Watertown, MA) beginning with a 5 min of denaturation step at 95∀C, followed by 40 cycles consisting of 30 seconds at 95∀C, 30 seconds at 60∀C and 1 min at 72∀C (final for 7 min).

To determine the species *C. gloeosporioides*, the primers ITS4 and CgInt were used. PCR reactions were conducted in a total volume of 20 σl, containing 40 ng of DNA, 2.0 mM $MgCl_2$, 200 σM each of dNTP, 0.5 σM each of the primers, 1 unit of Taq Polimerasa Promega⊇ (Promega Corp, Madison, WI), and 1X buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% Triton⊇ X-100). Amplifications were carried out in a PTC-100 thermal cycler (MJ Research, Inc, Watertown, MA) beginning with a 5 min of denaturation step at 95∀C, followed by 40 cycles consisting of 30 seconds at 95∀C, 30 seconds at 65∀C and1 min at 72∀C (final for 7 min). Amplification products were resolved by electrophoresis in a 1.2 % agarose gel, stained with ethidium bromide, and photographed under UV lighting.

Results and Discussion

The DNA extraction protocol used generated high quality DNA (Figure 1.22.1)

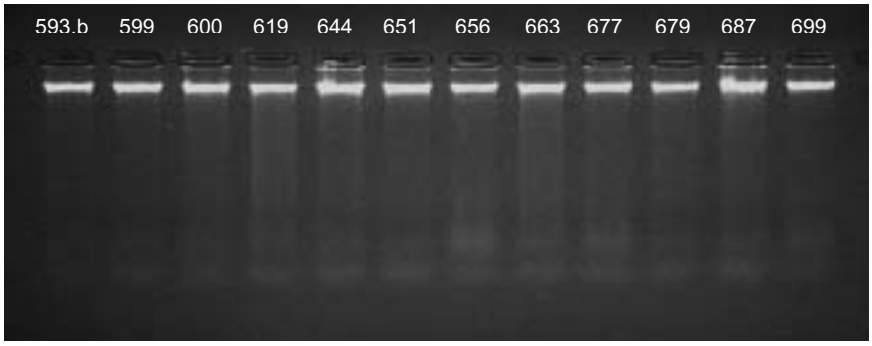

Figure 1.22.1. DNA samples isolated from isolates of *Colletotrichum* spp**.** originated from anthracnose lesions of citrus fruits in Colombia. The numbers at each lane are isolate numbers indicated in Table 1.22.1.

PCR amplifications: Many of the arbitrary primers tested so far resulted in limited polymorphisms (Figure 1.22.2). We are currently screening 40-50 more primers in an attempt to identify those that would generate polymorphism.

Amplifications with the primers CaInt2 – ITS4, indicated that all the isolates tested, with the exception of isolates 656, 677, 699 and the control isolate *C. gloeosporioides* (Cg), amplified a product with a 490 bp size that indicates that the species is *Colletotrichum acutatum*. On the other hand amplifications with the primers CgInt – ITS4 resulted isolates 656, 677, 699 as well as the control isolates generating a DNA product of 450 bp indicating that they all belong to the species *Colletotrichum gloeosporioides* (Figure 1.22.3). The results of the molecular identification of the isolates tested are presented in Table 1.22.1.
In addition to screening more arbitrary primers, we are currently working on implementing the amplified fragment length polymorphism (AFLP) technique for characterization of the pathogen population infecting the three fruit crops in this study.

Table 1.22.1. Isolates of *Colletotrichum* spp infecting citrus fruits used in this study.

	Isolate No.	Zone	Farm	Host	Host tissue	*Colletotrichum spp.*
1.	3	Armenia	El Piñal	Limón Tahití	Flower	
2.	5	Caicedonia	Danubio	Limón Tahití	Flower	*C. acutatum*
3.	6	Manizales	La Bejuca	Limón Tahití	Bud	*C. acutatum*
4.	14	Caicedonia	Danubio	Limón Tahití	Bud	*C. acutatum*
5.	55	Pereira	Catalina (FEDECAFË)	Limón Tahití	Bud	*C. acutatum*
6.	83	Pereira	Catalina (FEDECAFË)	Limón Tahití	Bud	*C. acutatum*
7.	100	Caicedonia	Maracaibo	Limón Tahití	Flower	*C. acutatum*
8.	107	Pereira	Catalina (FEDECAFË)	Limón Tahití	Bud	*C. acutatum*
9.	212	Caicedonia	Maracaibo	Limón Tahití	Flower	*C. acutatum*
10.	269	Pereira	Yarima	Limón Tahití	Bud	*C. acutatum*
11.	275	Pereira	Yarima	Limón Tahití	Bud	*C. acutatum*
12.	589	Villavicencio	El Refugio	Limón Tahití	Flower	*C. acutatum*
13.	590	Villavicencio	El Refugio	Limón Tahití	Flower	*C. acutatum*
14.	592	Cumaral	Las Brisas	Limón Tahití	Flower	*C. acutatum*
15.	593.a.	Cumaral	Las Brisas	Limón Tahití	Flower	*C. acutatum*
16.	593.b.	Cumaral	Las Brisas	Limón Tahití	Bud	*C. acutatum*
17.	594	Cumaral	Las Brisas	Limón Tahití	Bud	*C. acutatum*
18.	595	Cumaral	Las Brisas	Limón Tahití	Bud	*C. acutatum*
19.	596	Cumaral	Las Brisas	Limón Tahití	Bud	*C. acutatum*
20.	597	Cumaral	Las Brisas	Limón Tahití	Flower	*C. acutatum*
21.	599	Cumaral	Las Brisas	Limón Tahití	Bud	*C. acutatum*
22.	600	Villavicencio	El Refugio	Limón Tahití	Flower	*C. acutatum*
23.	611	Villavicencio	El Refugio	Limón Tahití	Flower	*C. acutatum*
24.	619	Villavicencio	El Refugio	Limón Tahití	Flower	*C. acutatum*
25.	644	Zona Bananera	La Inmaculada	Limón común	Flower	*C. acutatum*
26.	647	Zona Bananera	La Inmaculada	Limón común	Bud	
27.	651	Ciénaga	Las Margaritas	Limón común	Bud	*C. acutatum*
28.	654	Ciénaga	Las Margaritas	Limón común	Bud	
29.	656	Ciénaga	Las Margaritas	Limón común	Flower	*C. gloeosporioides*
30.	663	Ciénaga	Las Margaritas	Limón común	Flower	*C. acutatum*
31.	677	Zona Bananera	La Inmaculada	Naranja Tangelo	Leaf	*C. gloeosporioides*
32.	679	Zona Bananera	La Inmaculada	Limón común	Bud	*C. acutatum*
33.	681	Ciénaga	Las Margaritas	Limón común	Flower	
34.	687	Zona Bananera	La Inmaculada	Limón común	Bud	*C. acutatum*
35.	699	Montenegro	Estancia	Naranja Valencia	Flower	*C. gloeosporioides*

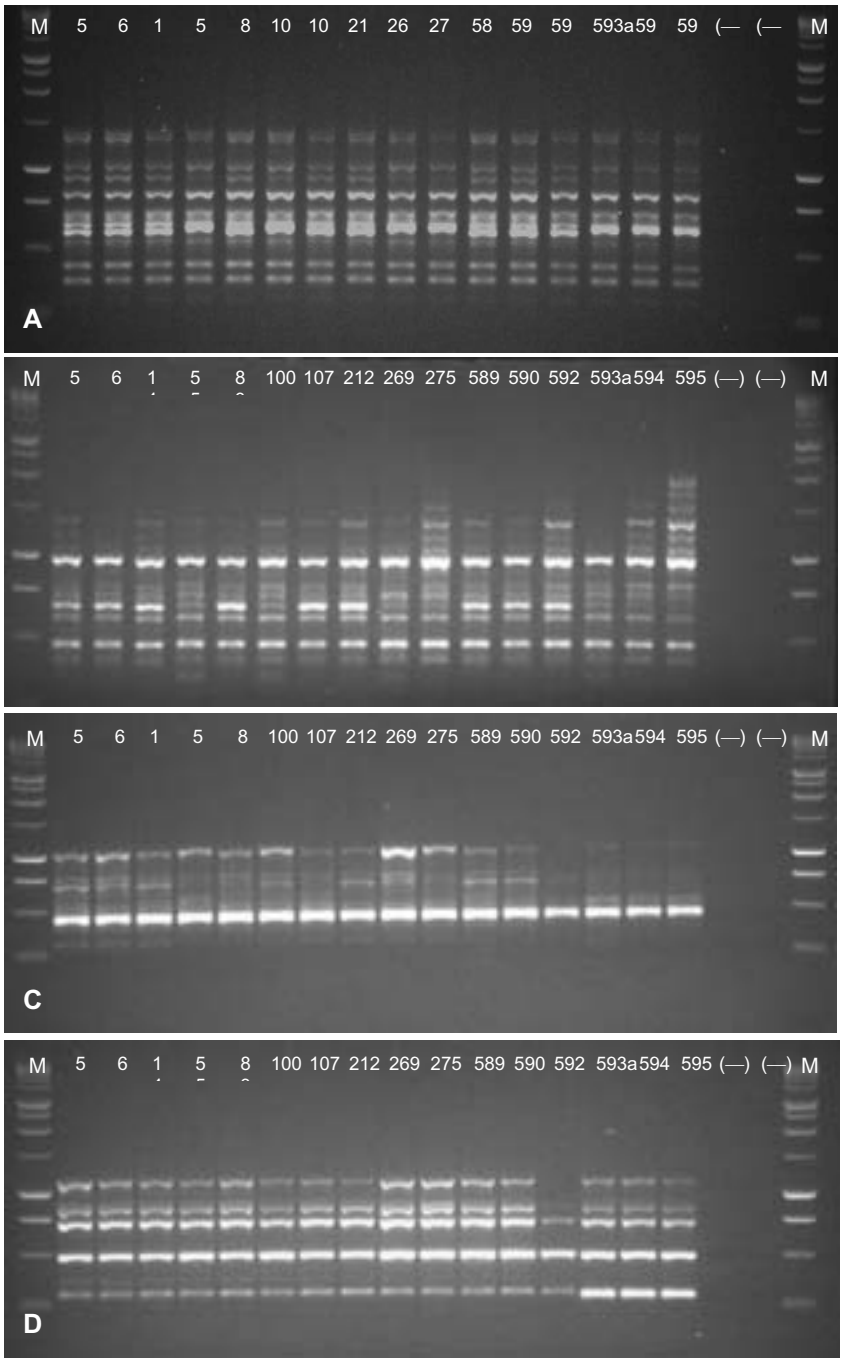

Figure 1.22.2. DNA samples from isolates of *Colletotrichum* spp**.** amplified with arbitrary primers **A,** primer A-02; **B,** primer AK-09; **C,** primer A-03; **D,** primer C-02. The numbers at each lane are isolate numbers described in Table 1.22.1. Lanes (∝), negative control without sample DNA; M, 1 kb marker.

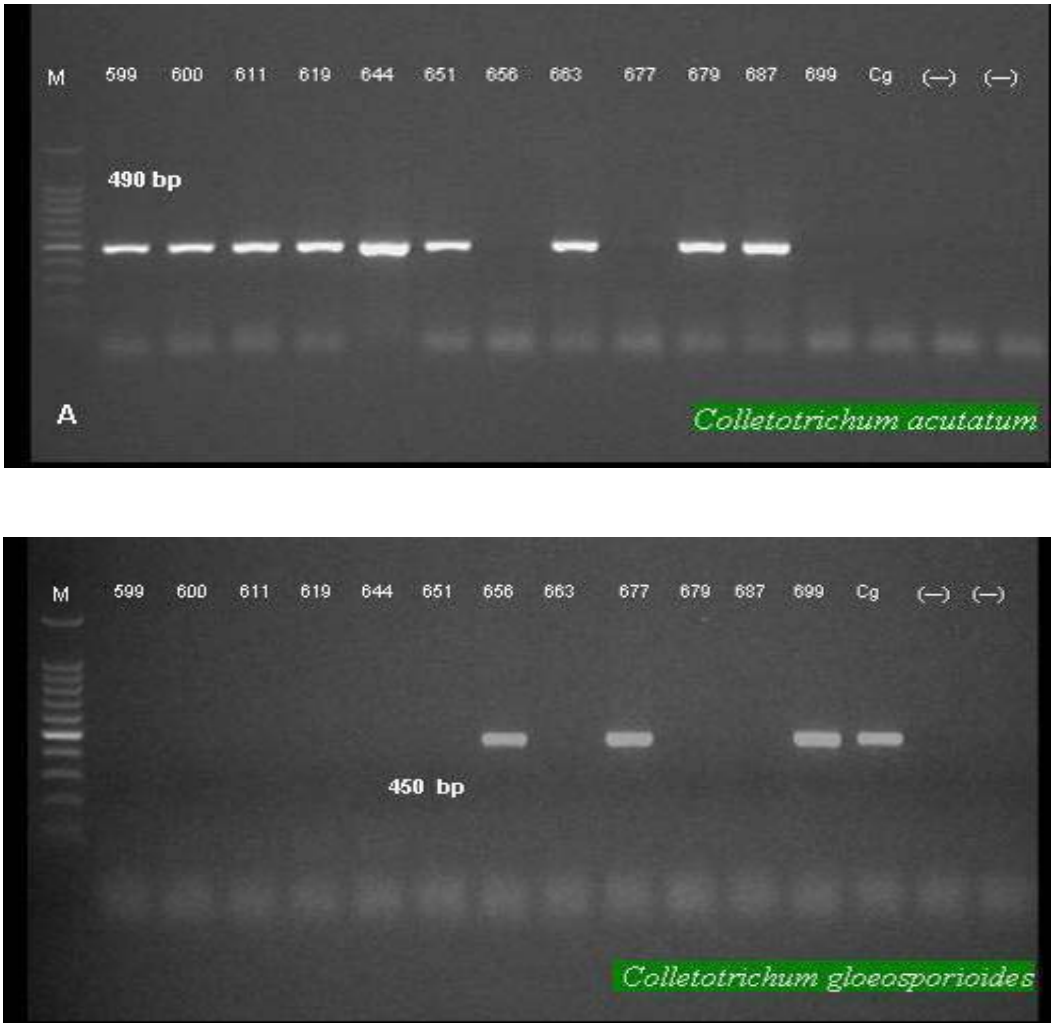

Figure 1.22.3. Taxonomic identification of isolates of *Colletotrichum* spp. using PCR. **A,** *Colletotrichum acutatum* (using primers CaInt2 and ITS4); **B,** *Colletotrichum gloeosporioides* (with primers CgInt and ITS4). The numbers at each lane are isolate numbers described in Table 1. Lanes (∝), negative control without sample DNA; M, 1 size marker; **Cg**, positive control *C. gloeosporioides*

Output 2: Pest-and-disease management components and strategies developed for key crops.

Activity 2.1. Levels of resistance to important insect pests confirmed in bean progenies

Contributors: C. Cardona, J. F. Valor, J. M. Bueno, A. Mejía, and M. Blair.

Highlights:

- Resistance to the bean weevil (*Acanthoscelides obtectus*) was identified in *Phaseolus vulgaris* x *P. acutifolius hybrids*
- New accessions and lines with insect resistance were identified

Rationale

A novel Double Congruity Backcross technique developed at CIAT has permitted the development of fertile insterspecific *Phaseolus vulgaris* x *P. acutifolius* (common x tepary) bean hybrids. These crosses are made using the tepary genotype NI576 (a genotype competent to *Agrobacterium*-mediated genetic transformation). Some of these crosses involve the tepary accession G 40199 an excellent source of resistance to the bean weevil, *Acanthoscelides obtectus* and leafhoppers. In previous years we identified several progenies containing both *P. vulgaris* and *P. acutifolius* cytoplasm with very high levels of antibiosis resistance to *A. obtectus* and a handful showing acceptable levels of tolerance to the leafhopper, *E. kraemeri*.

Materials and Methods

Depending on the amount of seed available, previously selected genotypes were multiplied in the field or under greenhouse conditions. The seed was then utilized to screen the different nurseries for resistance to *A. obtectus* in the laboratory. In most cases, genotypes were replicated four to five times. Levels of infestation varied form 2 to 3 mature eggs per seed. Percentage adult emergence and days to adult emergence were recorded. In some cases, individual seeds were tested using a level of infestation of two mature eggs per seed. Tests for resistance to *E. kraemeri* were conducted in the field under high levels of natural infestation, usually with 3-4 replicates per genotype in randomized complete block designs. Evaluations for resistance include damage scores and bean production ratings, insect counts, damage counts; in a few cases, yields, and yield components.

Results and Discussion

Acanthoscelides obtectus: In 2005, emphasis was placed upon the reconfirmation of resistance in previously selected progenies. Seeds of resistant hybrids selected in 2004 were multiplied in the greenhouse. The materials were then tested in replicated nurseries. As shown in Table 2.1.1 all but five of the hybrids turned out to be resistant. Resistance in some cases was as high as that of G 40199, the original resistant parent.

Table 2.1.1. Levels of resistance to *Acanthoscelides obtectus* in selected F_5 – F_7 hybrid progenies derived from interspecific *Phaseolus vulgaris* x *P. acutifolius* crosses.

Code and generation	Cross	Percentage adult emergence	Days to adult emergence	Rating
Interspecific *P. vulgaris* x *P. acutifolius* hybrids with *P. acutifolius* cytoplasm				
GNVAV 200A9 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	21.9	59.1	Intermediate
GNVAV 200D21 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	50.9	56.5	Susceptible
GNVAV 200D22 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	50.2	55.7	Susceptible
GNVAV 200G16 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	51.4	57.5	Susceptible
GNVAV 200G17 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	44.2	58.4	Intermediate
GNVAV 200G18 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	46.7	53.7	Intermediate
GNVAV 200G19 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	54.5	56.6	Susceptible
GNVAV 200H5 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	25.3	61.8	Intermediate
GVV 110G F_6	{[(G40022 x N1576) x V5] x A3} x VS42-7	10.2	65.6	Resistant
GVV 110I F_6	{[(G40022 x N1576) x V5] x A3} x VS42-7	33.1	59.8	Intermediate
GVV 108N F_6	{[(G40022 x N1576) x V5] x A3} x VS42-7	19.4	63.9	Resistant
BWG 1F7 F_5	BW-1 FL x GKA-12 F_3 FB	10.3	64.8	Resistant
BWG 1F13 F_5	BW-1 FL x GKA-12 F_3 FB	76.1	51.7	Susceptible
BWG 1F14 F_5	BW-1 FL x GKA-12 F_3 FB	25.8	57.2	Intermediate
BWG 1F18 F_5	BW-1 FL x GKA-12 F_3 FB	21.4	61.3	Intermediate
BWG 5N1 F_5	BW-1 FL x GKA-12 F_3 FB	13.9	60.1	Resistant
BWG 5N4 F_5	BW-1 FL x GKA-12 F_3 FB	15.8	51.7	Resistant
BWG 6Y6 F_5	BW-1 FL x GKA-12 F_3 FB	18.7	62.2	Resistant
BWG 6Y15 F_5	BW-1 FL x GKA-12 F_3 FB	34.2	43.1	Intermediate
Checks				
G 40168	Susceptible *P. acutifolius* accession	82.2	43.1	Susceptible
G 25410	Susceptible *P. lunatus* accession	91.1	44.5	Susceptible
ICA Pijao	Susceptible *P. vulgaris* cultivar	94.9	32.8	Susceptible
G 40199	Resistant *P. acutifolius* accession	9.6	66.5	Resistant
G 25042	Resistant *P. lunatus* accession	1.5	76.0	Resistant

The important process of testing individual seeds to detect segregation and reconfirm resistance in interspecific hybrids continued in 2005. Results (Tables 2.1.2 and 2.1.3) showed that several of the genotypes tested do possess interesting levels of antibiosis resistance to *A. obtectus*. Seed multiplication of resistant genotypes is in progress.

We also tested several progenies derived from intraspecific crosses made in *P. lunatus.* These had been selected in 2004 for very high levels of resistance to *A. obtectus*. With one exception, all were highly resistant to the bruchid (Table 2.1.4)

Zabrotes subfasciatus (Mexican bean weevil): Resistance to the Mexican bean weevil (*Z. subfasciatus*) has been successfully incorporated into bean cultivars using a backcross breeding method that combines biochemical tests for the presence of arcelin and insect feeding bioassays. Preliminary observations suggested that the incorporation of arcelin in bruchid-resistant lines (coded RAZ) might affect yields. To test this hypothesis, selected RAZ lines and their recurrent parents were yield-tested under field conditions at CIAT headquarters.

Results (Table 2.1.5) suggest that the incorporation of arcelin does have a depressing effect on yields. Most RAZ lines tested yielded less than their respective recurrent parents. Differences in most cases were not significant but nevertheless important. Further testing is planned for 2006.

Table 2.1.2. Reconfirmation of resistance to *Acanthoscelides obtec*tus in pre-selected segregating hybrid progenies derived from single-seed selections performed in interspecific *Phaseolus vulgaris* x *P. acutifolius* crosses.

Code and generation	No. of seeds tested	Percentage adult emergence	Days to adult emergence	Rating
Hybrids				
GKVGAG 1B 4D F_6	18	18.5	63.4	Resistant
GKVGAG 1B 4D F_6	13	20.5	64.2	Resistant
GKVGAG 1B 4D F_6	25	23.6	66.0	Intermediate
GKVGAG 1B 4D F_6	18	0.0	NEa	Resistant
GKVGAG 1B 4D F_6	30	36.6	57.6	Intermediate
Mean 5 resistant selections	Variable	19.8	62.8	Resistant
Mean 15 susceptible selections	Variable	72.0	58.3	Susceptible
GKVGAG 1E 2C F_6	23	44.1	63.5	Intermediate
GKVGAG 1E 2C F_6	30	7.9	58.9	Resistant
GKVGAG 1E 2C F_6	11	0.0	NE	Resistant
GKVGAG 1E 2C F_6	36	24.8	61.0	Intermediate
GKVGAG 1E 2C F_6	30	47.7	61.1	Intermediate
GKVGAG 1E 2C F_6	36	27.0	59.9	Intermediate
GKVGAG 1E 2C F_6	18	0.0	NE	Resistant
GKVGAG 1E 2C F_6	30	34.9	59.0	Intermediate
GKVGAG 1E 2C F_6	40	26.3	61.3	Intermediate
GKVGAG 1E 2C F_6	28	27.8	61.7	Intermediate
GKVGAG 1E 2C F_6	60	28.6	57.7	Intermediate
GKVGAG 1E 2C F_6	28	19.1	58.3	Resistant
GKVGAG 1E 2C F_6	15	44.2	57.9	Intermediate
GKVGAG 1E 2C F_6	11	21.2	49.4	Intermediate
GKVGAG 1E 2C F_6	15	22.2	56.2	Intermediate
GKVGAG 1E 2C F_6	20	41.7	65.1	Intermediate
Mean 16 resistant selections	Variable	26.1	59.3	Resistant
Mean 40 susceptible selections	Variable	71.3	56.4	Susceptible
Checks				
G 40168[b]	45	80.1	44.3	Susceptible
G 25410[c]	45	91.1	44.5	Susceptible
ICA Pijao[d]	125	94.9	33.0	Susceptible
G 40199[e]	45	9.6	66.5	Resistant
G 25042[f]	45	1.5	76.0	Resistant

[a] N.E., no adult emergence from resistant seeds; [b] Susceptible *P. acutifolius* accession; [c] Susceptible *P. lunatus* accession; [d] Susceptible *P. vulgaris* cultivar; [e] Resistant *P. acutifolius* accession; [f] Resistant *P. lunatus* accession.

Table 2.1.3. Reconfirmation of resistance to *Acanthoscelides obtec*tus in pre-selected segregating hybrid progenies derived from single-seed selections performed in interspecific *Phaseolus vulgaris* x *P. acutifolius* crosses.

Code and generation	No of seeds		Percentage adult emergence	Days to adult emergence	Rating
	Tested	Resistant			
Interspecific *P. vulgaris* x *P. acutifolius* hybrids with *P. acutifolius* cytoplasm					
NNQLAC 1G F_3	17	16	5.0	58.3	Resistant
Interspecific *P. vulgaris* x *P. acutifolius* hybrids with *P. vulgaris* cytoplasm					
T7K2C19 F_4	17	13	8.7	63.7	Resistant
T7K2C21 F_4	10	5	30.8	60.6	Intermediate
T7K2C23 F_4	6	5	12.5	64.0	Resistant
T7K2C25 F_4	4	2	55.6	52.5	Intermediate
T7K2F61 F_4	2	1	25.0	91.0	Intermediate
Checks					
G 40168[a]	20	0	76.8	56.5	Susceptible
G 25410[b]	20	0	90.9	47.3	Susceptible
ICA Pijao[c]	20	0	94.7	34.4	Susceptible
G 40199[d]	20	14	9.3	87.0	Resistant
G 25042[e]	20	18	1.7	91.0	Resistant

[a] Susceptible *P. acutifolius* accession; [b] Susceptible *P. lunatus* accession; [c] Susceptible *P. vulgaris* cultivar; [d] Resistant *P. acutifolius* accession; [e] Resistant *P. lunatus* accession.

Table 2.1.4. Resistance to *Acanthoscelides obtectus* in selected *Phaseolus lunatus* progenies.

Code and generation	Percentage adult emergence	Days to adult emergence	Rating
V5 F_3	0.0	NEa	Resistant
V5 F_3	85.5	57.6	Susceptible
V5 F_3	0.0	NE	Resistant
V5 F_3	0.0	NE	Resistant
V5 F_3	0.0	NE	Resistant
V5 F_3	0.0	NE	Resistant
V5 F_3	0.0	NE	Resistant
V5 F_3	0.0	NE	Resistant
A6 F_4	33.3	57.5	Intermediate
A6 F_4	0.0	NE	Resistant
Checks			
G 40168[b]	67.4	45.2	Susceptible
G 25410c[c]	82.2	50.2	Susceptible
ICA Pijao[d]	90.0	32.1	Susceptible
G 40199[e]	1.1	77.0	Resistant
G 25042[f]	0.0	NE	Resistant

[a] NE, no adult emergence; [b] Susceptible *P. acutifolius* accession; [c] Susceptible *P. lunatus* accession;
[d] Susceptible *P. vulgaris* cultivar; [e] Resistant *P. acutifolius* accession; [f] Resistant *P. lunatus* accession.

Table 2.1.5. Yields of RAZ lines and corresponding recurrent parents. RAZ lines are selected for the presence of arcelin and high levels of resistance to the Mexican bean weevil.

Line or cultivar	Recurrent parent	Yield (Kg/ha)	Difference with respect to recurrent parent		Significance with respect to recurrent parent
			(Kg/ha)	Percentage	
RAZ 160	ICA Pijao	2148	68	3.1	ns[a]
RAZ 165	ICA Pijao	2014	202	9.1	ns
RAZ 166	ICA Pijao	1997	219	9.9	ns
RAZ 162	ICA Pijao	1947	269	12.1	ns
RAZ 159	ICA Pijao	1931	285	12.9	ns
RAZ 154	ICA Pijao	1893	323	14.6	ns
RAZ 161	ICA Pijao	1878	338	15.2	ns
RAZ 158	ICA Pijao	1833	383	17.3	ns
RAZ 157	ICA Pijao	1831	385	17.4	ns
RAZ 153	ICA Pijao	1827	389	17.6	ns
RAZ 156	ICA Pijao	1812	404	18.2	ns
RAZ 155	ICA Pijao	1793	423	19.1	ns
RAZ 164	ICA Pijao	1781	435	19.6	ns
RAZ 163	ICA Pijao	1779	437	19.7	ns
Mean backcrosses to Pijao		1890	326	14.7	ns
ICA Pijao		2216			
RAZ 34	Ex-Rico 23	1178	580	33.0	*
Ex-Rico 23		1758			
RAZ 190	Talamanca	2035	55	2.6	ns
Talamanca		2090			
RAZ 151	EMP 250	1970	0	0.0	ns
RAZ 152	EMP 250	1597	346	17.8	ns
Mean backcrosses to EMP 250		1783	160	8.2	ns
EMP 250		1943			
RAZ 63	EMP 175	1208	404	25.0	ns
EMP 175		1612			
RAZ 65	WAF 2	1641	0	0.0	ns
WAF 2		1545			

[a] ns, not significant; *, significant at the 5% level by Dunnett's test for comparing all treatment means with the mean of a control.

Leafhopper (Empoasca kraemeri): We continued the work on evaluation of interspecific *P. vulgaris* x *P. acutifolius* hybrids. Similar to the work with bruchids, these progenies were obtained by means of the Double Congruity Backcross technique developed at CIAT. We tested 53 progenies of crosses made with the tepary sources of resistance to leafhopper G 40019 and G 40036. Selected progenies and their reaction to leafhopper are shown in Table 2.1.6 In general, the best lines show an intermediate level of resistance comparable to that found in the tolerant check, ICA Pijao. It can also be said that resistance to leafhopper in interspecific hybrids is not as good as the resistance found in *P. acutifolius* accessions G 40036 and G 40019.

Table 2.1.6. Resistance to *Empoasca kraemeri* in selected progenies derived from interspecific *Phaseolus vulgaris* x *P. acutifolius* crosses.

Code	Damage scores[a]	Reproductive adaptation scores[b]	Yield (Kg/ha)		Percentage yield loss	Susceptibility index[c]
			Protected	Non-protected		
A99Y-15F2	5.5	6.3	1212	1109	8.5	0.7
A99Y-86F2	6.0	6.0	1270	1021	19.6	0.9
A99Y-90F2	5.7	6.3	1349	1153	14.5	0.7
TSC123	6.0	5.7	1019	917	10.0	0.8
EMP 558	6.2	6.3	1412	1216	13.9	0.7
EMP 586	4.5	6.3	1769	1655	6.4	0.5
ICA Pijao	5.7	7.7	1761	1380	21.6	0.8
BAT 41[d]	8.4	4.0	1237	752	39.2	1.3
LSD 5%	0.5	1.3	317	379	-	-

[a] On a 1-9 visual scale (1, no damage; 9, severe damage); [b] On a 1-9 visual scale (1, no yield, no pod formation; 9, excellent pod formation and filling, excellent yield); [c] Calculated with respect to the mean of the trial and the mean Pijao, the tolerant check; [d] Susceptible check.

Activity 2.2. Integrated soil fertility / Pest and disease management to address root rot problems in common beans.

Contributors: Mahuku, G, Jara C., Cortes L. (IP-1) Barrios E., Navia J., Asakawa N., Quintero J. (TSBF-CIAT)

Highlight:

- Mulching with green manures increased bean yields of susceptible bean cultivars and this increase was associated with a reduction in root-rot incidence and the increased soil nutrient availability.

Rationale

The application of organic residues as mulches has been found effective in improving soil quality by increasing nutrient and water availability as well as controlling soil-borne pathogens by stimulating natural antagonistic organisms and/or producing toxic organic substances. Sources of green manure have different decomposition rates that regulate soil moisture and temperature while influencing the release of nutrients and and secondary compounds. This is likely to have different effects on the balance and relative population sizes of harmful and beneficial organisms. Our overall objective was to evaluate the impact organic residues of different qualities have on the abundance and diversity of soil nematodes, pathogenic and arbuscular mycorrhizal fungi (AMF).

Materials and Methods

The experiment was established in Ultisols at the CIAT's Santander de Quilichao Research Station, on an area that had a history of high incidence of root rot pathogens and used for selection of resistant bean genotypes. These soils have been systematically fertilized in the past and thus presented no nutrient limitations to bean plants during the experimental period (pH=6.8, total C = 2.5%, Bray-P II = 45 ppm, Exch. Ca = 10 meq/100 g soil). Experimental plots (size: 6x3m) were planted with the root-rot susceptible bean variety A70 during 4 consecutive seasons and here we report results for the last two seasons (2004A,

2004B). Figure 2.2.1 shows the climatic conditions during the period under report for CIAT's Santander de Quilichao Research Station.

Experimental treatments were covered with three green manures of contrasting quality (Table 2.2.1): (a) rapidly decomposing *Tithonia diversifolia* (TTH); (b) intermediate rate of decomposition by *Cratylia argentea* (CRA); (c) slow decomposing *Calliandra calothyrsus* (CAL) at a rate of 6 ton ha^{-1}; and (d) control (no green manure added). The experiment was replicated five times. The experimental layout is shown in Figure 2.2.2 Soil samples (0-10 cm) were collected within rows during the cropping season including planting and harvesting.

Table 2.2.1. Quality parameters for organic residues added to the soil as mulch. *C* carbon, *N* nitrogen, *ADF* acid detergent fiber, *NDF* neutral detergent fiber, *HEM* hemicellulose, *L* lignin, *PP* polyphenols (Cobo *et al*., 2002, Biol. Fertil. Soils 36:87-92).

Treatment	C %	N	ADF	NDF	HEM	L	PP	C/N	L/N	PP/N	(L+PP)/N
CAL	49.4	2.65	43.7	63.2	19	14.	18.44	18	5.47	6.9	12.43
CRA	44.3	3.28	42.6	64.2	21	17.	4.78	13	5.40	1.4	6.86
TTH	38.8	3.93	25.2	26.6	1	4.	8.65	9	1.16	2.2	3.36

Figure 2.2.2. Layout of the experimental plot at CIAT's Santander de Quilichao Research Station.

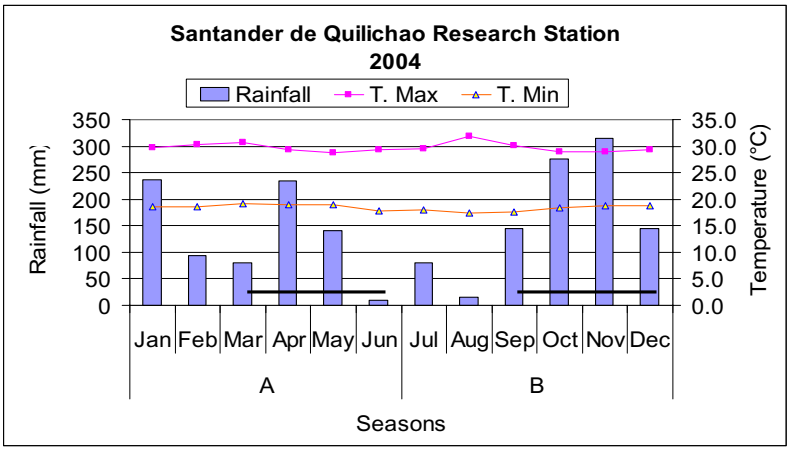

Figure 2.2.1. Climatic diagram during 2004 of CIAT's Santander de Quilichao Research Station. Two dark solid lines represent cropping seasons in 2004A and 2004B respectively.

Immediately after planting, the experimental plots were covered with three green manures of contrasting quality (Table 2.2.1): (a) rapidly decomposing *Tithonia diversifolia* (TTH); (b) intermediate rate of decomposition (but greater soil cover due to leaf morphology) by *Cratylia argentea* (CRA); (c) slow decomposing *Calliandra calothyrsus* (CAL) at a rate of 6 ton ha^{-1}; and (d) control (no green manure added). The experiment was replicated five times. Soil samples (0-10 cm) were collected during the cropping season, including at planting and harvesting. Samples were collected within rows and between rows, to measure the effect of the rhizosphere of bean plants on the soil biota studied.

Evaluation of soil microorganisms: Soil nematodes were extracted by density centrifugation with sucrose solution followed by careful separation by feeding habit. Abundance and diversity of soil pathogenic fungi were studied by serial dilution of soil and plating in selective media: PCNB for *Fusarium* and MA for *Macrophomina* and *Rhizoctonia*, complemented by evaluation of incidence of disease on susceptible plant genotypes. Arbuscular mycorrhizal fungi spores were extracted using density centrifugation with sucrose solution and hyphal lengths estimated using the membrane filtering technique (Miller & Jastrow, 1990, Sol Biol. Biochem. 22: 579-584). In addition, bean yield data was collected to estimate the overall effect of green manure application on crop productivity.

Results and Discussion

Bean yield and root-rot incidence following green manure treatments: Application of green manures consistently resulted in greater bean yields than the control largely as a result of lower root-rot incidence caused by *Macrophomina* (Figure 2.2.3). Significant yield differences among green manure treatments were only observed in the relatively drier 2004A season and CRA showed greatest differences with respect to the control. CAL consistently generated lower root-rot incidence than the control probably as a result of their slow decomposition (Stone *et al*., 2001, Soil Sci. Soc. Am. J. 65:761-770.) and high polyphenol content (Cobo *et al*., 2002, Biol. Fertil. Soils 36:87-92). Effective soil cover due to leaf architecture in CRA was beneficial during 2004A but actually led to increased incidence in 2004B, when soil moisture was greater, and resulted in lower relative yields. Application of TTH in 2004A showed low impact on incidence that contrasted with large incidence reduction during 2004B that was linked to highest relative bean yields. Soil analysis at the end of the 2004B season showed significantly higher total

C in CAL&CRA, total N in CRA&TTH, Bray-II P in CRA,TTH,CAL and exc. K in TTH while CON consistently presented the lowest value.

Changes in abundance and diversity of studied soil organisms: The mean abundance of *Fusarium* (Fus) in 2004B was about 1200 cfu g^{-1} soil and was not significantly affected by green manure treatment. *Macrophomina* (Mac), on the other hand, showed greater incidence in CRA and that is consistent with greater incidence observed in bean plants as shown in Figure 2.2.4. Nevertheless, despite high incidence of *Macrophomina* observed on infected plants we found relatively low abundances in the soil samples analyzed. Satellite experiments have been initiated to explore potential reasons for the limited detection of *Macrophomina* in the soil.

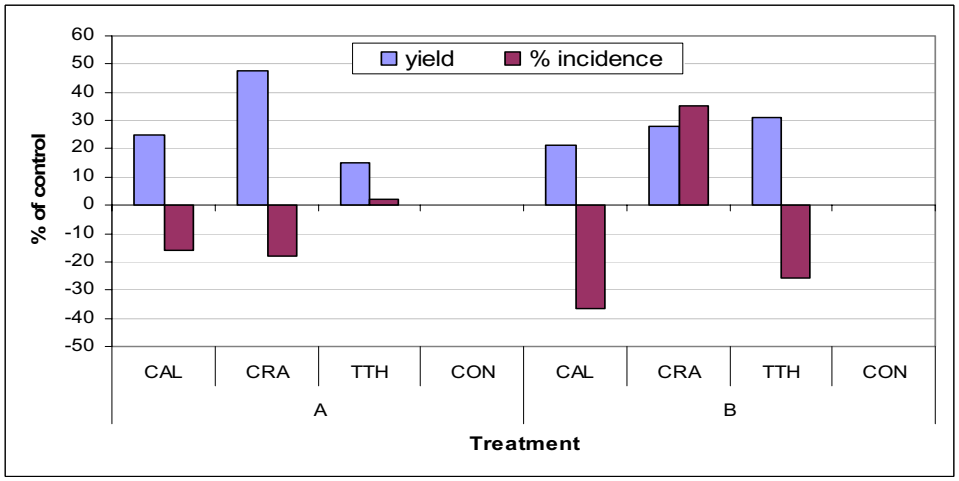

Figure 2.2.3. Crop yield and incidence of root rots of the bean genotype A70 expressed as a percent of the control treatment.

Abundance and diversity of nematodes: TTH consistently presented greater abundance of nematodes across both cropping seasons with values close to twice that of the control (Figure 2.2.5). This is likely a result of the high quality of tissues derived from this nutrient scavenging plant and their contribution to soil nutrient availability (Cobo *et al*., 2002, Biol. Fertil. Soils 36:87-92). Our results corroborate the quick response by bacterial feeding nematodes to increasing biological activity associated with nutrient additions to soil and faster decomposition rates. During 2004 greater differences existed between green manure treatments likely a result of the impact of different decomposition rates on soil moisture dynamics. Also a considerable overall reduction was observed in CON in 2004A but predatory and omnivorous nematodes populations were restored in 2004B when greater soil moisture was available.

Abundance of Arbuscular Mycorrhizal fungi (AMF): Mean AMF hyphal lengths were consistently higher in TTH compared to other treatments across both seasons (Figure 2.2.6). TTH has been previously reported to have high levels of colonization by AMF (Sharrock *et al*., 2004, Mycorrhiza 14:103-109.) and also grow on decomposing TTH shoots and leaves. Conversely, total AMF spore results were inconsistent and suggest the need for future analysis of isolated spores at the genus or species level.

Conclusions

Mulching with green manures generated increased yields of susceptible bean cultivars and this increase was associated with a reduction in root-rot incidence and the increased soil nutrient availability. This effect was most consistent in CAL and TTH, while in CRA it was only observed in the 2004A season. In the wetter 2004B season extended soil cover in CRA probably resulted in excess soil humidity/high root-rot incidence. The relative position of soil nematodes in the food chain and their rapid response to soil management, suggests their potential to influence through "ecological linkages" the population sizes of their food sources (i.e. fungi, bacteria, etc.). Having found greater abundance of fungivorous nematodes and AMF hyphae in TTH suggests no preferential feeding on AMF but rather on other soil fungi. Further studies in this long-term experiment will help testing this hypothesis.

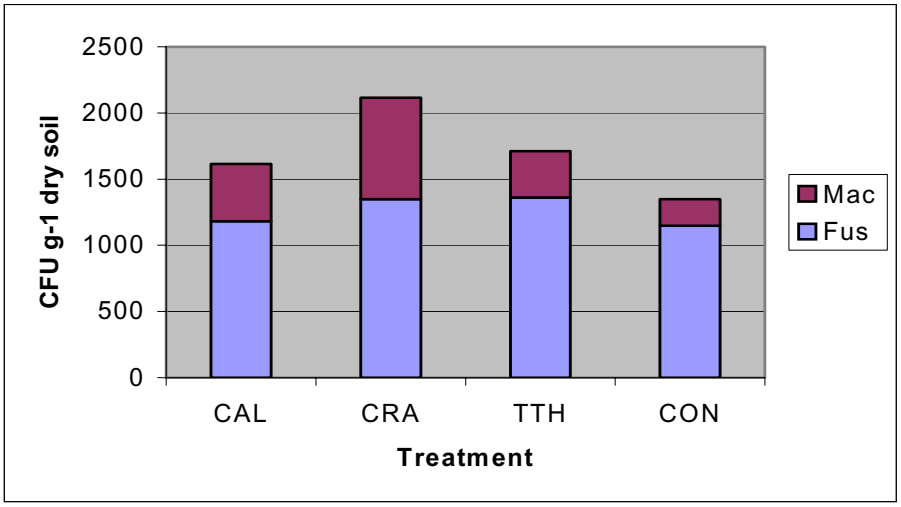

Figure 2.2.4. Abundance of *Fusarium* and *Macrophomina* across experimental treatments in 2004

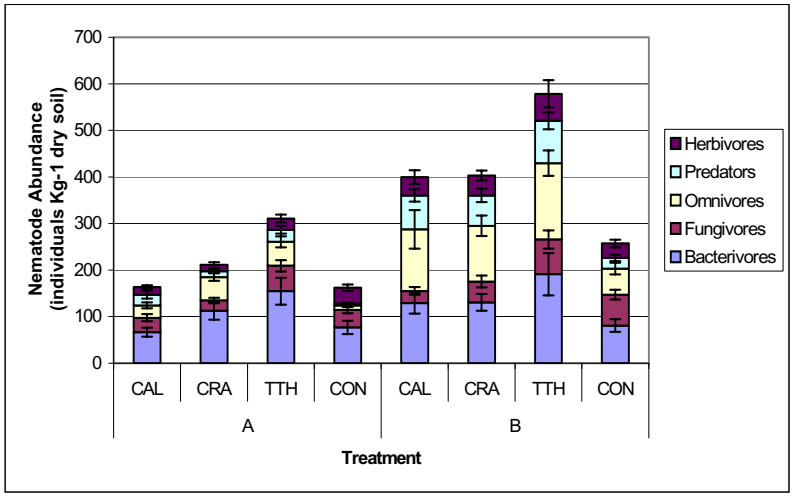

Figure 2.2.5. Abundance of soil nematodes discriminated by feeding habit across experimental treatments in 2004

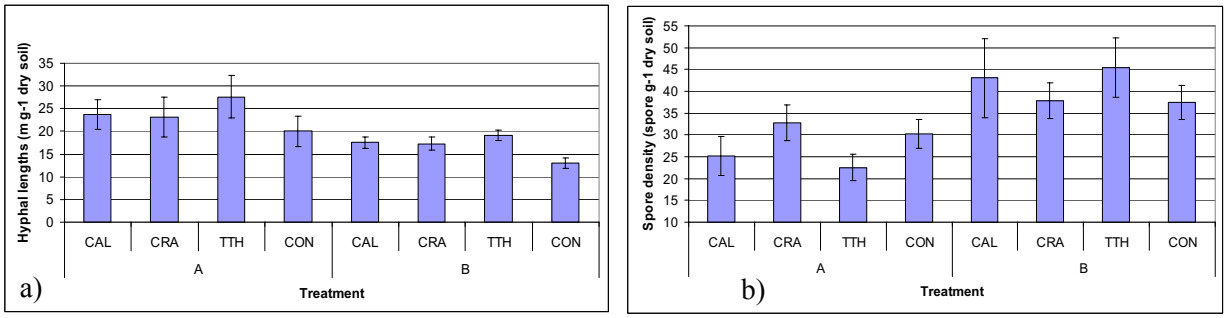

Figure 2.2.6. Activity and abundance of AMF estimated by a) hyphal lengths and b) spore counts in 2004.

Activity 2.3. Developing germplasm with multiple resistances to diseases in beans

Contributors: R. Buruchara, P. Kimani, R. Otsyula (graduate student), S. Sebuliba, A. Male and C. Acam**,** S. Beebe and M. Blair.

Highlights:

- Several bean lines with multiple resistance to Pythium root rot and ALS have been selected and will be distributed to different countries for multi-locational evaluations. Some have already been distributed to Kenya and Malawi

- A SCAR marker associated with ALS resistance gene in Mex 54 has been successful used in selection for ALS resistance

- A collaborative mechanism has been set up to facilitate national partners to integrate application of marker assisted selection (MAS) in their breeding program.

- Several elite lines and germplasm were screened for the presence or absence of I-gene. Donor parents were identified and a breeding program initiated to improving resistance in selected useful materials against BCMNV.

Rationale

In Sub-Saharan Africa, beans are produced both for home consumption and for the market. Beans can contribute to a healthier diet being a major source of proteins and a potentially a good source of iron and zinc for a majority of resource poor communities. Great market potential exists for different market classes of beans. Market expansion for existing products is one means to encourage farmers to produce and market larger crop surpluses. This is significant given that bean production in Africa is almost entirely in the hands of resource poor farmers, usually women. However, achieving food security, nutritional and economic goals is limited in part by the effects of diseases on productivity and quality of the produce. Genetic improvement is considered the most appropriate strategy for poor resources farmers to overcome these constraints. Last year, we reported progress made in improving resistance against two major bean diseases; angular leaf spot (ALS) and Pythium root rots in commercial and adapted bush and climbing bean cultivars. This year we continued selections from segregating populations and lines focusing on seed types and resistance to Pythium root rot, ALS and bean common mosaic virus.

Materials and Methods

Several F_2 derived F_4, F_5, and F6 lines were evaluated at Kawanda for resistance to Pythium root rot, angular leaf spot, common bacterial blight and bean common mosaic virus.
Evaluation for BCMV and BCMNV resistance was done in the field at Kawanda Research Institute, Uganda using natural infection. A BCMV infected crop (through seed) of G 2333 was grown as a border crop and after every 10 test lines.

A key source of resistance to Pythium root rot, RWR 719, suffers from black root when exposed to bean common mosaic necrotic virus (BCMNV) because it carries the "*I*" gene. Lines were also screened for the presence or absence of *I*-gene on the basis of SW13 marker. This was to determine those that may have inherited the "*I*" gene and which would require protection. DNA was extracted from young trifoliate leaves of 2-week old plants using ammonium acetate method. The trifoliate leaves were ground with the aid of sterile sand in an extraction buffer consisting of Tris- sodium chloride, EDTA, proteinase K and SDS. The resulting slurry was incubated at 65C for 1 hour. Ammonium acetate was later added, the DNA precipitated using isopropanol and the resulting pellet washed with 70%ethanol. The yield was determined using a quantifying ladder (Bioline). Each PCR reaction consisted of 5ng of the DNA in a 12.5µl reaction volume consisting of 0.2mM dNTPS, 2mM magnesium chloride, 1XPCR buffer, 0.3mM of each of the primer and 0.1units of Taq polymerase. Each amplification cycle consisted of the following steps: 15 seconds at 94 C, 30 seconds at 53 C, and 1 minute at 72. After 35 cycles the samples were subjected to a final extension for 7min at 72 C and kept at 4 C. Amplicons were resolved on 1.2% agarose gel stained with 10mg/ml Ethidium bromide and the gel subsequently immersed in 0.5XTBE. Electrophoresis was performed at 70V and bands visualized under UV light and the image captured on a digital camera mounted on a computer.

Mex 54 has been shown to be resistant to most races of *Phaeoisariopsis griseola* in Africa. It has been used in several crosses as a key source of resistance against angular leaf spot. The nature of resistance has been characterized. A RAPD marker $OPE4_{709}$ that had been shown to be linked to a resistance gene in Mexico 54 was converted to a SCAR marker. The utility of the latter was validated and applied in marker assisted selection. Young trifoliate leaves from 14-day old plants were used. The protocol described for BCMV was also used except for the annealing temperature. Each amplification cycle consisted of the following steps 15 seconds at 94C, 30 seconds at 65C, and 1 minute at 72. After 35 cycles the samples were subjected to a final extension for 7min at 72C and kept at 4C.

Results and Discusión

Selection of recombinant inbred lines (RILs) for improved resistance to Pythium root rot: In addition to progenies selected last year from 14 crosses, 76 new progenies were selected this year from 6 additional crosses (Table 2.3.1.)

Last year progenies were selected from 16 crosses, 14 of which were used to develop populations by bulking all single plant selection harvested. Those with enough seed were planted at two sites (Rubaya and Kawanda) and others at only one site, giving priority to Rubaya (Table 2.3.1). Selection was based on plants with good architecture, seed types, yield and maturity in addition to resistance to Pythium root rots. A total of 242 progenies were selected from 13 crosses in Rubaya, while 320 progenies were selected from 10 crosses in Kawanda (Table 2.3.2).

Table 2.3.1. F_5 lines resistant to *P. ultimum* derived from F_2 resistant progenies by pedigree method. Kawanda, 2004A.

Pedigree	Line Code	Number of resistant progenies
GLP 585 x AND 1055	RF RO2-24	27
CAL 96 x AND 1062	RF RO2-35	13
URUGEZI x RWR 719	RF RO2-41	1
URUGEZI x SCAM 80CM/15	RF RO2-43	24
URUGEZI x AND 1055	RF RO2-44	7
URUGEZI x AND 1062	RF RO2-45	4

The trials in Rubaya were heavily infected with BCMNV, and therefore in 2005A they were re-grown at Kawanda under high incidences of BCMNV and selection was based on the resistance to this disease. From this, only 28 progenies were selected from 10 crosses (Table 2.3.2).

Selection of recombinant inbred lines (RILs) for improved resistance to angular leaf spot: Over 500 recombinant inbred lines (RILs) developed for resistance to Pythium root rots were also evaluated for ALS under field conditions at Kawanda. Selection was based on a score of less than 3.9 in the 1-9 CIAT disease severity scale. A total of 139 progenies were selected.

Multiple resistance crosses selected for ALS and Pythium root rot: Of the 285 F_6 progenies derived from crosses to combine resistance to Pythium and angular leaf spot, 196 were screened for Pythium resistance under screenhouse conditions. Of these, 85 had a score Ò3.9 on the 1-9 CIAT scale (Table 2.3.3).

Table 2.3.2. F_2 derived F_5 populations selected for Pythium root rot and BCMV, Uganda**.**

Crosses	Rubaya (2004B)		Kawanda (2004 B)		Rubaya lines selected in Kawanda (2005 A)	
	Lines planted	Lines selected	Lines planted	Lines selected	Lines planted	Lines selected
GLP-2 x RWR 719	200	17	1400	64	17	1
GLP-2 x MLB-49-89A	200	32	456	30	32	3
GLP-2 x SCAM 80CM/15	200	18	434	25	18	0
GLP-2 x AND 1055	23	7			7	1
GLP-2 X AND 1062	200	33	172	22	33	0
GLP 585 x RWR 719	200	39	780	7	39	4
GLP 585 x MLB-49-89A	182	55			55	1
GLP 585 x AND 1055		13			13	0
GLP 585 x AND 1062		10	126	10	10	1
CAL 96 x RWR 719		24	128	24	24	4
CAL 96 x AND 1055		11	45	11	11	0
URUGEZI x RWR 719		18	160	18	18	2
URUGEZI x MLB-49-89A		43	200	43	43	5
URUGEZI x AND 1062			200	0		6
Total selected		242		320		28

Single plant progenies selected for resistance to Pythium root rot and ALS are shown on Table 2.3.4. These consist of materials representing different market classes and types including the Calima, black, carioca, pink, grey, purple and cream; and large, medium and small seed types. It is interesting to note that 65 % of the selected progenies are of the preferred Calima seed types.

Application of (OPE4 $_{709}$) marker to select for ALS resistance and SW13 to determine presence of I-gene: Screening for I-gene on the 285 progenies using SW13 marker was initiated and is still on-going. Of these, 251 have already been screened, and 22 of have the "I" gene only, while, 122 were positive for the OPE4$_{709}$ SCAR marker, and 31 were positive for both the OPE4$_{709}$ SCAR and *I-* gene markers (Table 2.3.5).

Table 2.3.3. Evaluation (screenhouse for Pythium and OPE4 $_{709}$ SCAR marker for ALS) of progenies derived from crosses to combine Pythium and ALS resistance, Kawanda, 2005A.

	Pythium Evaluations				ALS Evaluations		
		Disease severity (on 1-9 CIAT scale) Screenhouse					
Combined Crosses	No. of progenies evaluated	1	1.1-3.9	Ò8.9	Number of progenies evaluated	Selected in screen house	Selected by marker (SCAR OPE4 $_{709}$)
CAL 96 x RWR 719) x CAL 96 x MEX 54)q	96	2	38	56	106	24	44
CAL 96 x SCAM 80 CM/15) x CAL 96 x MEX 54)	64	2	35	27	129	68	20
CAL 96 x MLB 49-89 A) x CAL 96 x MEX 54)	36	0	8	28	50	21	7
Total	196	4	81	101	285	113	71

Table 2.3.4. Selections made combing Pythium and ALS representing different seed types from crosses in 2005

Pedigree	Line Code	Selected plants	Seed color	Seed size within a market class	No of progenies within a seed size
CAL 96 x RWR 719) x CAL 96 x MEX 54)	RF RA 02-1	13	black	small	6
CAL 96 x SCAM 80 CM/15) x CAL 96 x MEX 54)	RF RA 02-6	5	black	medium	12
CAL 96 x RWR 719) x CAL 96 x MEX 54)	RF RA 02-1	33	calima	small	38
CAL 96 x MLB 49-89 A) x CAL 96 x MEX 54)	RF RA 02-3	41	calima	medium	47
CAL 96 x SCAM 80 CM/15) x CAL 96 x MEX 54)	RF RA 02-6	14	calima	large	3
CAL 96 x MLB 49-89 A) x CAL 96 x MEX 54)	RF RA 02-3	1	carioca	small	1
CAL 96 x RWR 719) x CAL 96 x MEX 54)	RF RA 02-1	1	cream	small	7
CAL 96 x MLB 49-89 A) x CAL 96 x MEX 54)	RF RA 02-3	21	cream	medium	14
CAL 96 x SCAM 80 CM/15) x CAL 96 x MEX 54)	RF RA 02-6	1	cream	large	2
CAL 96 x MLB 49-89 A) x CAL 96 x MEX 54)	RF RA 02-3	1	grey	small	1
CAL 96 x RWR 719) x CAL 96 x MEX 54)	RF RA 02-1	1	pink	medium	1
CAL 96 x SCAM 80 CM/15) x CAL 96 x MEX 54)	RF RA 02-6	1	pink	large	1
CAL 96 x RWR 719) x CAL 96 x MEX 54)	RF RA 02-1	1	purple	medium	1
Total selected plants		134			

Key to seed sizes, small =22 gm >, medium =23 – 35 gm, Large = 36 – 45 gm Market class colors are based on the primary colors.

Table 2.3.5. F_5 progenies screened for the SW13 and OPE_{709} SCAR marker.

Pedigree	Number of families with		
	$OPE4_{709}$ SCAR marker only	**SW13 marker only**	**$OPE4_{709}$ and SW13 markers**
F1 (CAL 96 x RW 719) x F1 (CAL 96 x MEX 54)	35	11	16
F1 (CAL 96 x RWR 719) x F1 (CAL 96 x BAT 332)	0		
F1(CAL 96 x MLB-49-89A) x F1(CAL 96 x MEX 54)	65	9	14
F1(CAL 96 x SCAM 80- CM/15) x F1 (CAL 96 x BAT 32	0		
F1(CAL 96 x MLB-49-89A) x F1(CAL 96 x BAT 332)	0		
F1(CAL 96 x SCAM 80CM/15) x F1CAL 96 x MEX 54)	22	2	1
Total	122	22	31

Development of backcross (BCs) populations with Pythium root rots resistance: Parallel to the development of RILs, a backcrossing program to transfer resistance into popular market class types (backgrounds) was initiated in 2004. Twenty (20) backcross populations were generated. Currently $BC_{s5}F_3$GLP2 x RWR 719 with 111 progenies have been given to partners in Kakamega and was also planted in Kawanda for seed multiplication and homogeneity studies. The other populations are in the field as bulk progenies and will be planted as single plant selections to develop progenies in the coming year. They will be made available to partners for heterogeneity test and for selecting lines of interest (resistance and farmer preferences) under different environments.

In addition, BC_{S3}F1 was advanced to BC_{S4}, and BC_{S3}F2 was advanced to F3 with the objective of trapping seed with the background of the susceptible parent before BC_{S4}

Seed distribution: A total of 557 RILS (F_6) were developed last year. This year the first set of RILS was distributed to Namulonge in Uganda, Kakamega in Kenya, and Malawi for multi-location evaluation with farmer participation (Table 2.3.6).

Conclusion: Considerable progress was made in efforts to improve resistance against two key constraints in Africa. The application of markers in progeny selection introduced a dimension which will enhance the efficiency and effectiveness of the process and allows the pyramiding of key traits.

Table 2.3.6. Seed distribution of recombinant inbred lines in 2005[a].

Crosses	Namulonge	Kakamega	Malawi
GLP-2 x RWR 719	29	29	14
GLP-2 x MLB 49-89A	31	31	14
GLP-2 x SCAM 80CM/15	5	5	2
GLP-2 x AND 1055	5	5	
GLP-2 X AND 1062	6	6	1
GLP 585 x RWR 719	7	7	2
GLP 585 x MLB 49-89A	54	54	43
GLP 585 x SCAM 80CM/15			
GLP 585 x AND 1055	12	12	3
GLP 585 x AND 1062	12	12	5
CAL 96 x MLB 49-89A	5	5	
CAL 96 x AND 1062	11	11	3
URUGEZI x RWR 719			
URUGEZI x SCAM 80CM/15	1	1	1
URUGEZI x AND 1055	1	1	1
URUGEZI x AND 1062	3	3	1

Activity 2.4. Application of MAS in progeny evaluation and selection within improved populations of beans

Contributors: R. Buruchara, A. Male (CIAT), A. Namayanja (NARO), A. Musoni (ISAR), G. Mahuku, and P. Kimani

Rationale

Marker-assisted selection (MAS) offers advantages in speeding and improving effectiveness of breeding and in pyramiding desired genes into commercial backgrounds. This is potentially useful within NARS breeding programs where breeders are taking on more challenging and complex breeding objectives and schemes. Since most NARS breeding programs have limited access to facilities for applying MAS, a mechanism is being developed to facilitate adoption and adaptation of MAS as a routine procedure in breeding programs through a regional collaborative (networking) approach. Networking has been successfully used to transfer technology, germplasm and experience horizontally across a number of countries. The objective of this activity was to facilitate NARS programs to integrate and apply MAS for certain traits of interest where markers have been identified, using the biotechnology laboratory at Kawanda, Uganda.

Mex 54 is resistant to most races of *Phaeoisariopsis griseola* found in Africa and has been extensively used to improve resistance of commercial but susceptible varieties. In previous years we showed that a RAPD primer OPE-04 is associated and segregates with a single resistant dominant gene in Mex 54 (detected when using race 63-39 of *P. griseola*). This RAPD marker has been converted to a SCAR marker ($OPE4_{709}$) offering possibilities for more reliable results in the application of MAS to select resistant progenies for one of the priority traits in Africa.

There is also a growing interest to detect the presence or absence of "*I*" gene in progeny selections particularly where BCMNV is important and in using *bc-3* in breeding for resistance against BCMV and BCMNV.

Materials and Methods

Resistance to angular leaf spot (ALS), Pythium root rots and bean common mosaic necrotic virus are some of the traits of interest in varietal improvement programs of Rwanda, Uganda and Kenya. With the availability of markers and facilities at Kawanda, Uganda, there has been interest to introduce MAS, focusing on certain traits to ensure that priority genes are incorporated into the final products.

Advanced lines developed (F5 and F6) to improve or combine resistances which include angular leaf spot in commercial popular varieties were assayed for markers associated with resistance to the disease to verify the presence of desired alleles. Twenty-six lines were assayed from Rwanda and 75 from Uganda. DNA extraction and assaying were done as described in 2.4.1(i) above. Resistance to ALS was assayed using the SCAR marker associated with resistance gene in Mex 54 (Figure 2.4.1).

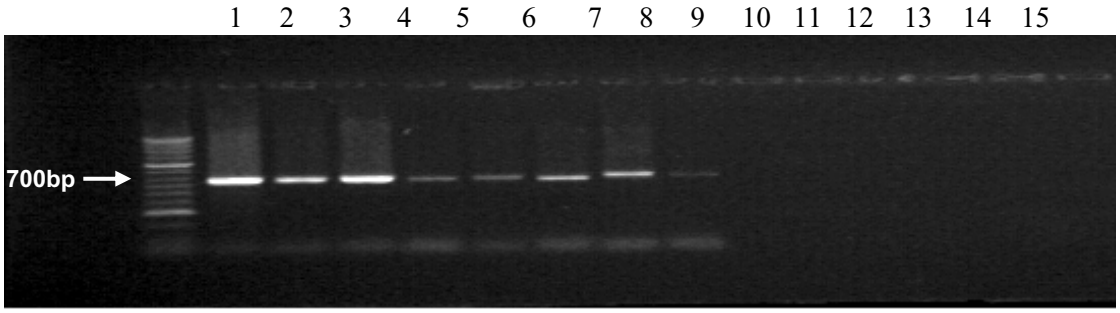

Figure 2.4.1. Results of PCR analysis using the SCAR primer $OPE4_{709}$. Sample 1 is Mex 54 while sample 15 is CAL 96. Samples 2 to 8 show presence of marker while lines and 9 to 14 show absence of the marker.

Results and Discussion

Out of the 26 lines assayed from Rwanda, 60% had the SCAR marker implying presence of the resistance gene. And of the 75 lines that have been assayed so far from Uganda, only 10 (13%) showed the presence of the marker (Table 2.4.1). The lines from Rwanda have previously undergone selection at the ALS hotspot in Rubona, and this probably explains the relatively high frequency of lines that seem to have the resistance gene. Using MAS, it was possible in a short time to repeatedly evaluate and identify lines with resistance to ALS. This approach will be used not only to make further selections from the two countries but will be accessible to other bean networks member countries.

Table 2.4.1. Advanced lines from Rwanda and Uganda screened for ALS resistance using the $OPE4_{709}$. SCAR marker, Kawanda, Uganda.

	Number of Lines		
Line-Code	**Evaluated**	**With Marker**	**Without Marker**
Rwanda Lines			
Urugezi x Puebla x Mex 54	2	2	0
Ngwinurare x SCAM 80CM/15 x Mex 54	4	4	0
Umubano x Mex 54	2	2	0
Vuninkingi x Mex 54	3	3	0
Umubano x Mex 54	6	2	4
Urugezi x Puebla x Mex 54	1	1	0
Ngwinurare x Puebla x Mex 54	3	1	2
Vuninkingi x Puebla x Mex 54	1	0	1
Mexico 54	1	1	0
Vuninkingi	1	0	1
Umubano	1	1	0
Urugezi	1	0	1
Sub-total	**26**	**17**	**9**
Uganda, Namulonge Lines			
Mex 54 x (Kanyebwa x Mex 54)	5	2	3
K132 x (K 132 x Mex 54)	4	0	4
K 132 x Mex 54	10	2	8
Mex 54 x (K 132 x Mex. 54)	14	3	11
Urugezi x Mexico 54	9	0	9
K 20 x Mexico 54	1	1	0
POA 2 x (Mex 54 x G 2333)	17	2	15
K 132 x (Mex 54 x G 2333)	1	0	1
AND 1055 x (Mex 54 x G 2333)	1	0	1
RWR 2075 x (Mex 54 x G 2333)	12	0	12
RWR 719 x (Mex 54 x G 2333)	1	0	1
Sub-total	**75**	**10**	**65**

Activity 2.5. Improvement of bean common mosaic necrotic virus resistance in common bean (*Phaseolus vulgaris)*

Contributors: R. Buruchara, A. Male, S. Ssebuliba, P. Kimani, (CIAT), Gerardine mukeshimana (UNR-Rwanda), A. Namayanja (NARO-Uganda), Reuben Otsyula (KARI-Kenya), and Geoffrey Tusiime (Makerere University - Uganda), M Blair and P. Kimani, (CIAT)

Rationale

Bean common mosaic virus (BCMV) and bean common mosaic necrosis virus (BCMNV) are the most serious viral diseases affecting production of common beans in Africa. A dominant resistance ("*I")* gene confers resistance to a wide range of BCMV strains by inducing a hypersensitive resistant (HR) reaction

to BCMV. With the widespread occurrence of BCMNV in Africa, the potential of genotypes addressing different constraints or consumer objectives, cultivars and released varieties possessing the *I*-gene are at times never fully utilized and in some cases the *I*-gene becomes a liability. For example there are several lines or varieties which have been released and adopted by farmers because of their value against some of the most limiting factors in bean production in the region. These include RWR 719, RWR 2075, RWR 1946 and RWR 1873 which are all very good against root rots and have preferred seed characteristics. Unfortunately, these lines carry the *I*-gene limiting their usefulness to areas having no BCMNV strains or in seasons when occurrence or spread or BCMVN is low. To provide stable, broad-based resistance, a suitable strategy is to protect the *I*-gene in varieties by combining the *I*-gene and race-specific resistance genes (typically *bc-2*[2] or *bc-3*) and also to introgress resistance into key materials that neither have *I*-gene nor any type of resistance to BCMNV. The aim of this effort is to develop high yielding varieties of major market classes that combine and/or are resistant to bean common mosaic necrotic virus (BCMNV). Given that markers for *I* and *bc-3* genes have been identified and mapped, marker assisted selection has potential to speed and enhance effectiveness of the breeding effort.

Materials and Methods

This is a collaborative effort between three countries; Rwanda, Kenya and Uganda. The bean programs in the three countries have either released, or have used the *I*-gene materials in their breeding programs. This implies that several advanced, elite, promising and commercial bean lines have the *I*-gene that may causes the lethal black root reaction with BCMNV. The initial effort was therefore to characterize advanced, elite, promising and released lines for the presence or absence of *I*-gene and initiate protection of *I*-gene in elite and commercial varieties.

Characterization of advanced/elite/promising and released lines for the presence or absence of I-gene, which results in black root against BCMNV: Over 1025 genotypes consisting of advanced lines, released varieties and other germplasm where the *I*-gene was possibly incorporated or whose one of the parent may have contained the *I*-gene were assembled from National Agricultural Research Organization (NARO), Uganda; Kenya Agricultural Research Institute (KARI), ISAR, Rwanda, ECABREN, Regional Breeding programme, Nairobi and CIAT, Kawanda. Preliminary field screening for the presence of *I*-gene was done on about 400 entries at Senge, Kawanda, a hotspot for both BCMV and BCMNV. Lines were categorized as either susceptible with a mosaic reaction, *I*-gene or no symptoms. All plants that showed no symptoms (mosaic or black root) to BCMNV (may include possible escapees) were selected for confirmatory evaluations in the laboratory using the SW13 marker for the presence of *I*-gene. DNA was extracted and assayed as described in section 2.1.1(*i*) for the presence or absence of the *I*-gene. Genotypes of interest having the *I-gene* will be protected while lines or varieties without the *I*- gene will have *I and bc-3* genes incorporated and lines that combines *Ibc-3* genes will be evaluated by farmers or used in other breeding programs.

Protection of I-gene in elite and commercial varieties from the lethal hypersensitive reaction induced by BCMNV: The focus is to protect released varieties and advanced lines, which have desirable agronomic or consumer traits but have the *I*-gene. Seed of sources of resistance (USWK-6, TARS VAR -7, USCR-7, USCR-9, TARS VAR -1) having combinations of *I* and *bc-3* genes were obtained from Miklas (USA) and increased in the greenhouse. Introgression was started with RWR 719 and MLB-49-89A, both important varieties in root rot management. Introgression of resistance genes in more commercial varieties will be done when adequate seed of donor parents is harvested.

Results and Discussions

Field screening for resistance to BCMNV and black root: Three groups of germplasm and lines were evaluated for resistance to BCMV and presence of *I*-gene under field conditions at Senge, Kawanda Agricultural Research Institute. Each entry was planted in plots of 2 rows, 3m long. Plots were separated by 50cm and spreaders were planted after every 10^{th} plot. Plant reaction was assessed on the basis of presence or absence of mosaic and black root at 4 different times during the growth period of the plant.

Of the nearly 400 entries constituting mainly of lines from NARO, Uganda, CIAT Kawanda and Rwanda, about 53 % showed black root (Table 2.5.1) implying a high proportion of materials with *I*-gene.

Table 2.5.1. Reaction of groups of germplasm to natural infection of BCMNV under field conditions.

Germplasm / Lines	Source	Number of entries	Number showing black root
Bush lines	NARO-Uganda	170	94
PPB IYT	CIAT-Uganda	63	38
Climbers (complex crosses)	ISAR, Rwanda	72	28

Screening germplasm for I-gene using SW13 marker: Entries that had been evaluated in the field were also evaluated using the SW 13 marker to compare and confirm observations made in the field. DNA extraction and assaying was done as described above on about 100 lines (Figure 2.5.1) The use of markers was more efficient as it was possible to detect lines that had escaped detection in field evaluations. Out of 65 lines derived from 11 climbing bean population developed in Rwanda, 35 were found to be positive for the *"I"* gene. This allows the bean program to make decisions on how to proceed with these lines given the fact that, much as it conditions resistance to the non-necrosis inducing strains of the virus, the presence of the (*I*) gene renders varieties carrying it to be susceptible to the necrosis inducing strains that are very prevalent in Africa.

Screening of donor materials for the presence of I and bc-3 genes: The objective of this activity was to screen potential donor materials and verify the presence of *I* and *bc*-3 genes. Five donor varieties (expected to contain both the *I* and *bc*-3 genes) namely: USWK-6, TARS VAR-7, USCR-7, USCR-9, TARS VAR-1 were grown in the screenhouse at Kawanda. CAL 96 was included as a negative check. DNA extraction and amplification was done as described above. Amplification was done using SW13 and ROC 11 primers. With the SW13 primer, all donor varieties gave one band while the check variety CAL 96 gave none (Figure 2.5.2). When the ROC 11 primer was used, the reverse was true; the susceptible variety (CAL 96) gave a band while all the rest showed none (Figure 2.5.3). This confirmed presence of *I* and *bc*-3 genes in theses varieties but which lacked in the susceptible variety CAL 96.

Introgressing bc-3 gene into commercial cultivars with I-gene through a backcrossing breeding programme: Because of inadequate seed of donor resistant parents, this activity was initiated by making crosses with two *I*-gene parents RWR 719 and MLB-49-89A. The F_1 populations were generated and will be advanced to F_2 generation by selfing. The F_2 populations will then be evaluated for the presence of *I* and *bc-3* genes.

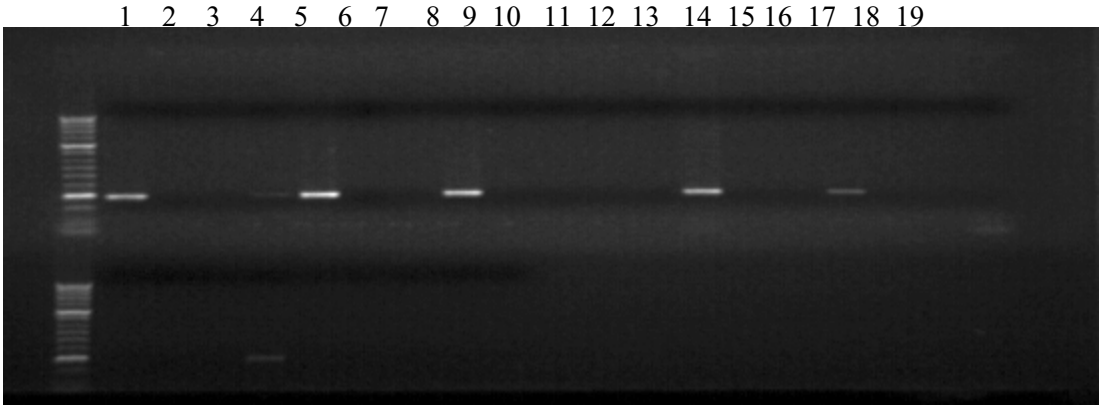

Figure 2.5.1. Left lane is 100 bp ladder. Lane 1 is a positive control variety (USWK 6). Lanes 5, 8, 13, 16 are positive for *I*-gene while the rest are negative.

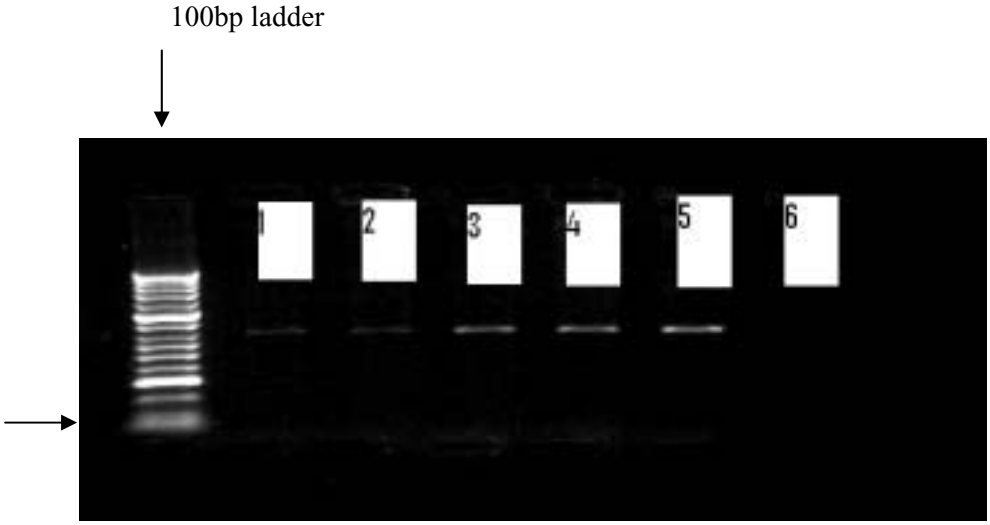

Figure 2.5.2. Amplification of donor parents using SW 13 primer. Lane 1= USWK-6; Lane 2 = TARS VAR-7; Lane 3 = USCR-7; Lane 4 = USCR-9; Lane 5 = TARS VAR-1 and Lane 6 = CAL 96.

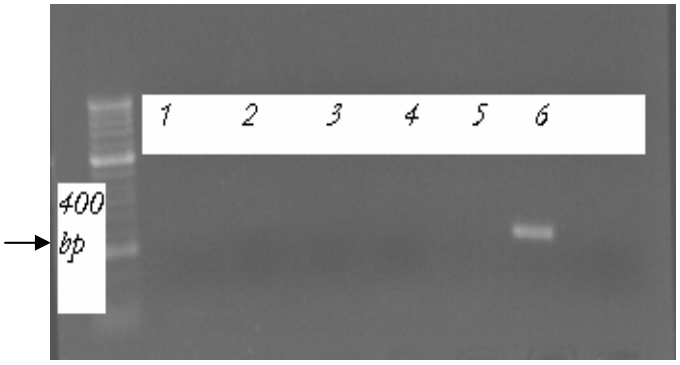

Figure 2.5.3. Amplification of donor parents using the ROC 11 primer: Lane 1 = USWK 6; 2= USCR 9; Lane 3 =TARS VAR 1S; Lane 4 = USCR7; Lane 5 = TARS VAR-1 and lane 6 = CAL96.

Activity 2.6. Improvement of beans (*Phaseolus vulgaris*) for resistance to Fusarium root rot (*Fusarium solani* f.sp. *phaseoli*) in large seed size

Contributors: C. Mukankusi, R. Buruchara, C. Acam, S. Musoke (CIAT), R. Melis, W. de Milliano (University of Kwa-Zulu-Natal), and Steve Beebe

Rationale

Fusarium root rot disease caused by the fungus *Fusarium solani* fsp. *phaseoli* (FSP) is an important soil-borne fungal disease causing Fusarium root rot on common bean (*Phaseolus vulgaris* L.). A number of control measures exist but the use of resistant varieties is considered the most economical method for small-scale bean growers in East Africa. Generally, large seeded bean varieties are preferred in central, south and western Uganda while small seeded bean varieties are more preferred in eastern and northern Uganda. There is also a general preference for large seeded beans at market level. However, large seeded bean varieties generally lack resistance to FSP. Resistance has been reported in small and late maturing varieties having less preferred seed characteristics. The objective of this study is to develop large seeded market class beans in Uganda that are resistant to *Fusarium solani* fsp. *phaseoli.*

Materials and Methods

Characterisation of potential sources of resistance: Germplasm (57 lines) obtained from CIAT Colombia including documented sources of resistance to FSP, Uganda land races (37 lines), Pythium root rot nursery (57 lines), 21 lines from Potchefstroom Agricultural Research Council, South Africa and 10 from the University of Kwa-Zulu Natal were characterized basing on seed color, size, hypocotyl color, flowering and maturity dates, flower color and growth habit.

Isolation and pathogenicity of FSP isolates: A protocol adopted from Burgess et al. (Burgess *et al*., 1994, Lab. manual for Fusarium research, 3rd Edition, pp 133) with several modifications was followed in the isolation of the pathogen from plant tissues. Inoculum was prepared by growing the FSP isolate on sorghum seed in 500ml inoculum bottles for 2-3 weeks. Three isolates, 2 (FSP 1 and FSP 3) which had the characteristic blue centre and white margin and slow growing described for pathogenic FSP and macro-conidia shape (Tusiime, 2003, PhD. Thesis. Makerere University Kampala, Uganda, pp113) and one with white mycelia and fast growing (FSP 2) were tested. The isolates were added to the trays at a rate of 500ml of sorghum inoculum per 74 x 42 x11.5 cm^3 tray of sterilised soil. Seed of three local and popular varieties K20, Kanyebwa and K132 that are susceptible to FSP were planted in the infected soil. Two documented sources of resistance G4830/ Rio Tibagi, and G 4495/ Porrillo Sintetico varieties and MLB-49-89A (partially resistant), were also planted as checks. The trays were watered every two days to provide ideal environment for disease development.

Screening potential sources of resistance: Local and introduced potential sources of resistance were evaluated at Kawanda Agricultural Research Institute as described above and were assessed on the basis of incidence and disease severity (percentage of plant tissue showing symptoms of FSP; where 0% = no visible symptoms and 100% approximately the whole of the hypocotyl and root tissues have lesions). Plants with severity scores of 0-15% were regarded resistant; 15-25% as moderately resistant; 26-49% as moderately susceptible, and ∅50% as very susceptible.

Development of crossing blocks: A recurrent selection with backcrossing program with the aim of introgressing FSP resistance in some popular but susceptible varieties, i.e., K20, Kanyebwa and K132 was developed. Possible sources of resistance RWR 719, NABE 7C and Umgeni were used in a North Carollina Mating Design II. Genetic parameters, i.e., inheritance of FSP resistance (hf^2), inheritance of

seed size (hs^2) will be determined. Mechanism of resistance to FSP will also be investigated as well as effect of some cultural practices, i.e., inoculation techniques, depth of seed sowing, level of soil compaction, level of soil moisture, presence of nematodes and time of planting, to the expression of resistance to FSP by different cultivars. The study will also shade more light on the genetic relationship between seed size and FSP resistance.

Results and Discussion

Pathogenicity Testing: Among the three isolates, FSP-3 was the most virulent with severities ranging from 27.5% (MLB-49-89A) to 85% on K132 and was hence selected for use in screening for resistance and subsequent activities (Table 2.6.1).

Table 2.6.1. Reaction of 5 common bean lines to 3 FSP isolates (% of root and hypocotyls tissues affected)

FSP Isolates	MLB-49-89A	K132	K20	G4830/ Rio Tibagi	G 4495/ Porrillo Sintetico
FSP-1	32.5	65.0	63.5	76.0	85.0
FSP 2	0.0	5.0	5.0	0.0	0.0
FSP 3	25.7	85	78.1	All dead/rotten	All dead/ rotten

Screening for resistance to FSP: Seventy lines have been screened so far for resistance to FSP which include 49 from the CIAT Pythium root rot nursery, and 21 from Potchefstroom. Of these 18 lines gave severities levels of less than 40%. The line MLB-49-89A from the CIAT Pythium root rot nursery showed the highest degree of tolerance to FSP and will be used as a source of resistance. Thirty-five accession from Cali, Colombia and 10 lines from the University of Kwa-Zulu-Natal gave susceptible reactions (severities Ó50%).

Development of FSP crossing block: Blind crosses were made between CAL 96, Kanyebwa, RWR 719, Vuninkingi and Umgeni using a North Carolina Mating Design II. Reciprocal crosses were also made. F1's (18 crosses) from the crossing block have been planted to obtain F2 seed for screening against FSP. F1 seed will also be screened for resistance to FSP before backcrossing to the large seeded parents.

Actiivity 2.7. Pathogenicity of *Pythium spp* and effects of management options for root rots on crops grown in association with beans in southwest Uganda

Contributors: R. Buruchara, V. Gichuru, (graduate student), S. Buah, C. Acam (CIAT), and F. Opio (NARO)

Rationale

Bean is one of the crops grown under the intensive agricultural system in southwest Uganda. Others include sorghum, maize, sweet potatoes, Irish potatoes, bananas and peas. Crop rotation in the strict sense is rare. Dominance of crops in the field shifts according to season. Rotations commonly practiced include beans-maize-sorghum, beans-maize-beans and beans-Irish potato/maize-sweet potato. Maize and sorghum are also intercropped with beans and/or Irish potatoes such that the bean crop appears in the field

season after season. However, of all these crops, beans are most affected by root rots. In recent years this has resulted in the decline in bean production in the area. *Pythium* is the major pathogen causing severe root rot on beans (Mukalazi, 2004, Pathogen variation and quantification of *Pythium* species in bean fields in Uganda, PhD dissertation, pp 59-60, Makerere University, Kampala, Uganda) and can result in severe losses up to 100 % in some seasons. Other pathogens, which also display root rot symptoms, include *Fusarium spp.* and *Rhizoctonia solani,* which occur singly, or in complexes with other pathogens including *Pythium spp. (*Tusiime, 2004, Variation and detection of *Fusarium solani* f.sp. *phaseoli* and quantification of soil inoculum in common bean fields, PhD. Thesis, Makerere University, Kampala, Uganda, pp 113). *Pythium spp* are known to have a wide host range. This study is part of broader investigations to determine the role of other crops in a bean-based cropping system to the overall root rot problem in southwestern Uganda. Its focus is to determine the pathogenicity of some *Pythium* species associated with major crops found in the bean based systems.

Materials and Methods

Last year we reported initial results of pathogenicity studies which we continued with this year. Four *Pythium* species pathogenic to beans (*P. ultimum, P. chamaehyphon, P. pachycaule and KAK 5B)* were artificially inoculated on four crops commonly associated with beans namely: sorghum, millet, maize and peas. Autoclaved millet (100 g) was mixed with 200 ml of water in a 500-ml bottle which was used to raise the fungi. After two weeks of incubation, the infested millet was mixed with pre-sterilised soil at a ratio 1:8 v/v in wooden trays. Maize, sorghum, millet and peas were planted in two rows of twelve plants and replicated in three trays. Bean varieties CAL 96 and RWR 719 were used as susceptible and resistant checks respectively. Cumulative emergence and plant stand was recorded one week after germination. Three weeks after germination, plants were assessed for any root symptoms that may be associated with *Pythium* infection using the CIAT 1-9
scale.

Results and Discussion

Emergence of various crops determined one week after planting was not significantly different from the control in all the three trials. However, the Pythium isolates significantly affected the level of disease on CAL 96, sorghum and peas (Tables 2.7.1 & 2.7.2). Maize and millet were not significantly affected by the *Pythium* isolates and gave disease scores of less than 5.0. Sorghum and peas were significantly affected by bean pathogenic *Pythium spp* thus suggesting that these two crops could be playing a role in the root rot problem in southwestern Uganda where they are major intercrops or rotation crops. The root dry matter yield of the various crops did not show any significant difference from the control.

Table 2.7.1. Pathogenicity of bean pathogenic *Pythium* species on CAL 96, maize, sorghum, millet and RWR 719.

Treatment	Trial one					Trial two				
	CAL96	Maize	Sorghum	Millet	RWR 719	CAL96	Maize	Sorghum	Millet	RWR 719
Control	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
MS61	6.59***	2.77***	5.67***	1.67ns	1.1ns	6.89***	3.75***	8.03**	1.0ns	2.86***
VIH 2A	4.55***	4.33***	4.11***	5.16***	1.0ns	7.41***	4.61**	8.00**	1.0ns	2.5***
KAK 5B	3.97***	1.89ns	6.44***	1.89ns	1.05ns	8.92***	2.83**	7.39**	1.0ns	2.75***
JM 29 A	5.92***	2.00***	6.00***	3.22***	1.13ns	5.92***	3.5**	6.91**	1.0ns	2.97**

Table 2.7.2. Pathogenicity of bean pathogenic *Pythium* species on CAL 96, peas and RWR 719

Treatment	Trial one			Trial two		
	Disease severity			Disease severity		
	CAL96	Peas	RWR 719	CAL96	Peas	RWR 719
Control	2.2	2.2	2.3	1.0	1.0	1.0
MS61	5.1***	5.4***	2.8ns	4.86***	8.36***	2.97***
VIH 2A	5.7***	5.9***	2.6ns	5.44***	7.72***	2.75***
KAK 5B	4.4***	4.5***	2.7ns	4.72***	5.75***	2.64***
JM 29 A	6.1***	7.6***	2.9***	5.39***	7.31***	4.33***

ns - not significantly different (P>0.05)
***-significantly different (P<0.05) by Dunnett test

Activity 2.8. Effects of management options for bean root rots on crops grown in association with beans in southwest Uganda

Contributors: R. Buruchara (CIAT), W. Ocimati (graduate student), F. Opio (NARO), M.A. Ugen (NARO)

Rationale

In southwestern Uganda beans are often grown in association with other crops, i.e., maize (79%), sorghum (52%), peas (46%), Irish potatoes, sweet potatoes and yams. At any one time there are at least three crops grown together. Some *Pythium* spp have been isolated from the roots of some of these crops (CIAT 2004, Africa: Bean Pathology. CIAT annual report 2004, pp 347 – 350). Besides, *Pythium* spp pathogenic to beans have been observed to cause disease on some crops grown in association with beans. To understand if crops grown in association or in rotation with beans play any role in the pathogen survival, inoculum density and severity of root rots in beans we continued with some of the studies we initiated last year to document the level of root rot in the major intercrops of beans in this region and to determine the effects of management options for bean root rots on crops grown in association with beans.

Materials and methods

The study was done on farmer fields in Rubaya sub-county, Kabale District in southwestern Uganda, a hotspot for bean root rots. Eight farmer fields with a history of bean root rot incidence and severity were selected on the basis of discussions, interviews and experiences of farmers.

Effect of root rots on major bean intercrops: Root rots were examined on three major intercrops of beans i.e. maize, sorghum and peas grown in a trial that also assessed different management options that are known to reduce root rots in beans. CAL 96, a susceptible bean cultivar was grown as a check.

Effect of management practices: The effects of four root rot management options i.e. farmyard manure, green manure (*Crotalaria)*, NPK fertilizer and a fungicide (Ridomil) previously known to have useful effects against bean root rots were evaluated on sorghum, maize, peas and beans. Farmyard manure and green manure (Crotalaria) were applied on a dry weight basis at a rate of 5t/ha. NPK fertilizer was applied at a rate of 50KgN/ha. Fungicide (Ridomil) was applied as slurry at a rate of 2.5 kg/ha. A plot with no soil amendment was used as a control. Qualitative data was obtained through field observations and photography. Quantitative data included plant stand and disease incidence at 18, 36, 54 and 72 days after planting (DAP) and disease severity at 36, 54 and 72 DAP. Disease severity was evaluated according to the CIAT nine-point scale where 1 is resistant and 9 susceptible. Other parameters included plant vigor and dry matter yield at 36 and 54 days respectively.

Results and Discussion

Effect of root rots on major bean intercrops: Root rot lesions were observed on all the crops indicating the presence of infection by root rot pathogens. Above ground symptoms could be seen on beans and sorghum at 18 days after planting. In beans, damping off at seedling stage, yellowing of leaves, stunted growth and, in severe cases, deaths of plants was observed. Infected roots had brown water soaked lesions and in the severe cases were rotten. Plants that survived to late stages were stunted, yellow and produced a few flowers and pods. Severely infected plots had a poor plant establishment. Sorghum plants exhibited stunted growth, purpling of leaves (Figure 2.8.1), shoot death, extensive tillering, prop root development, dark-red to black root lesions and ultimately a poor plant establishment. Purpling and yellowing seems to be due to nutrient deficiency probably excerberated by reduced root surface as a result of root rot damage. Tillering and prop root development are mechanisms developed by the plant for more water and nutrient uptake, thus its survival.

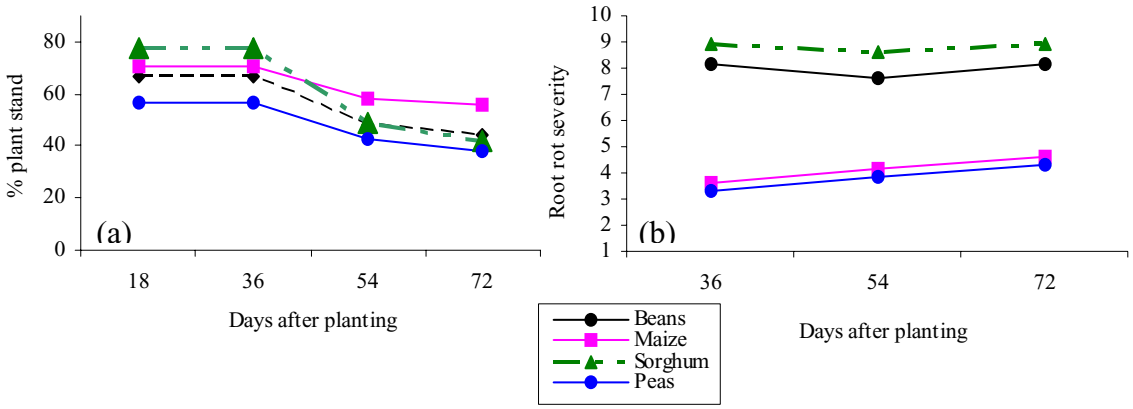

Figure 2.8.2. Plant stand during 2004B season (a), and root rot severities during 2004B season (b).

Plant death was observed on sorghum and beans during the season (Figure 2.8.2a). Similarly, higher lesion severity was recorded in sorghum (> 8) and beans (check) (> 7) than in maize (> 4) and peas (> 3) (Figure 2.8.2b).

Figure 2.8.1. Purpling in severely affected sorghum plants

The above results indicate that sorghum and beans were most affected and are hence more susceptible to root rots than maize and peas. In southwest Uganda, sorghum and beans are the major crops grown (in large acreage) in rotation with each and their susceptibility may indicate that they may be contributing to the root rot pathogen inoculum build-up in soil. However, the high level of susceptibility of sorghum to root rot was unexpected and these results imply that the effects of root rots on sorghum and its role in inoculum build-up may have been underestimated in the past.

Effects of management options on incidence and severity of root rots: The amendments improved crop tolerance to root rots early in the season. They improved crop survival, reduced root rot severity and increased dry matter relative to the control (Tables 2.8.1 & 2.8.2). Improved dry matter production and vigor under Ridomil is probably due to its protective effect against *Pythium* species. Plant recovery was evident in plots amended with GM, FYM and NPK. In addition, FYM, GM and NPK, enhanced root (mass) growth. FYM and GM improve soil physical properties, which probably enhance plant tolerance, and create conditions unsuitable for the root rot pathogens. Ridomil and farmyard manure were more effective resulting in lower incidence and severity in all seasons compared to other treatments. Though GM improved crop tolerance (increase in DM & vigor) it increased root rot incidence and severity early in the season (Table 2.8.1). This necessitates delaying planting to allow decomposition. Effectiveness of the management options varied with time in the season with Ridomil and FYM being more effective early in the season i.e. up to 36 days after planting, while GM from mid to late in the season (Table 2.8.1). From 54 days after planting no significant difference ($P>0.05$) in severity was observd in all the treatments.

Enhancing nutrient availability by addition of NPK also improved crop tolerance as it increased vigor and dry matter yield. NPK resulted in the highest vigor and dry matter yield (Table 2.8.2). This demonstrates the importance of adequate soil nutrient supply in enhancement of crop tolerance to root rot. Manipulation of the variation in effectiveness of the amendments can therefore be exploited for developing a management strategy combining two or more compatible control methods.

Thus using these options does not only contribute to the management of bean root rots, but is also beneficial to other crops. Similar amendment effects were observed in all crops except for GM and FYM in peas. FYM and green manure increased root rot incidence and severity in peas (Table 2.8.1). However, Farmyard manure (FYM) compensated for this effect as shown by the pea dry matter yield (Table 2.8.2).

Conclusion: The major intercrops/ rotational crops of beans in southwestern i.e. sorghum, maize and peas are affected by root rots. Sorghum like beans in this region is susceptible to root rots while maize and peas are tolerant. This suggests that these crops may be contributing to the root rot inoculum load in beans and focus needs to be given to root rot control in these other crops as well. More still, rotations involving beans and some crops such as sorghum may not be helpful in reducing bean root rots. The bean root rot management practices are equally useful to other crops in the system. Delayed planting after addition of organic amendments especially green manures improves their effectiveness against bean root rot pathogens. Therefore in developing management strategies for root rots, it would be advantageous to consider a systems' approach rather than a commodity. However, there is need to investigate the root rot organisms responsible for root rots of the major crops in this region. It is also important to investigate the role of other possible alternative hosts of root rot pathogens of beans apart from maize, sorghum and peas in this region; the effect of a wide range root rot management options; effects of serial application of green manures and compost manures and the socio economic aspects of using these management options in this region.

Table 2.8.1. Effect of soil amendments on root rot severity in beans, maize sorghum and peas from field trials in Rubaya, 2004B season.

Crop	Soil amendment	Days after planting (DAP)		
		Root rot Severity		
		36	54	72
CAL 96	Control	5.987	7.937	8.388
	Farmyard manure	5.750	7.350	8.150
	Green manure *(Clotalaria)*	5.699	7.455	8.186
	Ridomil	5.900	8.000	8.000
	NPK (17;17;17)	6.275	7.475	8.075
Maize	Control	3.913	4.750	5.737
	Farmyard manure	3.137	3.863	4.413
	Green manure *(Clotalaria)*	4.080	4.519	4.293
	Ridomil	3.038	3.625	4.425
	NPK (17;17;17)	3.775	4.050	4.338
Sorghum	Control	8.563	8.750	8.750
	Farmyard manure	7.050	8.625	9.000
	Green manure *(Clotalaria)*	8.042	8.693	8.954
	Ridomil	7.025	8.488	9.000
	NPK (17;17;17)	6.667	8.622	8.924
Peas	Control	3.100	3.737	4.450
	Farmyard manure	3.450	4.188	4.400
	Green manure *(Clotalaria)*	4.046	4.304	4.759
	Ridomil	2.550	3.588	4.175
	NPK (17;17;17)	3.212	3.475	3.825
CV%		**23.300**	**13.000**	**12.000**
LSD (0.05)		**0.526**	**0.788**	**0.778**

[1]Within columns, means with the same letter (s) and no letters do not differ significantly at P>0.05
[2]Season 2004B (September to January)
[3] Disease scale (1-9) 1 = no root symptoms; 3 = 10% of the hypocotyl and root tissues have lesions; 5= 25% of the hypocotyl and root tissues lesions 7= 50% the hypocotyl and root tissues have lesions and the root system suffers a considerable decay; 9= 75% or more of the hypocotyl and root tissues have lesions and the root system suffers advanced stages of decay and considerable reduction.

Table 2.8.2. Effect of bean root rot management practices on dry matter production (54 days after planting) for beans, maize, sorghum and peas from Rubaya, Kabale District, 2004 B season' g/ 5 plants for beans, sorghum and peas; g/3 plants for Maite.

Treatment	Beans	Maize	Sorghum	Peas
Control	9.71b	12.71c	2.81b	15.04 c
Farm Yard Manure	12.81b	28.65 a	6.24a	25.30 a
Green Manure	14.85a	20.19 b	3.44a	14.66 c
Ridomil	12.20b	14.65 c	2.70b	16.15 bc
NPK	13.21a	23.08 b	3.29a	19.76 b

[1]Means of the same letter(s) and no letters within columns do not differ significantly at P≤0.05
[2]Season 2004B (September to January)
Adaptation scale (Abawi and Pastor-Corrales, 1990, CIAT publication, 114 pp), 1- most vigorous; 9- least vigorous.

Actiivity 2.9. Assessment of the potential of candidate organism as a biocontrol agent against Pythium root rot

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Rationale

Root rots of beans have become increasingly important in several areas of eastern and central Africa. Occurrence and severity are associated with high intensity of bean production, and where intensification of land use has resulted in reduced crop rotation and fallow periods, leading to decline in soil fertility and a build up of soil pathogen. Management strategies considered mainly include use of resistant varieties, crop rotation, cultural practices and the use organic and inorganic amendments. Biocontrol agents offer an option that could contribute in strategies to manage *Pythium* root rot. The objective of these studies is to identify and evaluate the interactions between naturally occurring soil-borne disease-moderating organisms that have potential in the management of *Pythium* root rot.

Materials and Methods

Laboratory screening: Seventeen isolates (MS46, MS49, MS11, MS47, MS34, DFD47, KIS4, MS15, MS27, MS6, MS66, KLE3A, MS61 (*P. ultimum*), VIH4, KB4, MS10 (*Mortierella*) and KB14) were screened against each other for their potential use as biocontrol agents. The antagonistic activity of the isolates were determined in a dual culture assay in which opposite ends of the potato dextrose agar (PDA) plate was inoculated with an isolate and incubated at 24°C. Qualitative data of interactions (inhibition or lack of it) were recorded after 48 h of incubation. Four isolates (KB 14, KB 4, VIH 4 and MS 61) showed mutual inhibition on contact with MS10 whereby the space between the two organisms was small but clearly marked (Figure 2.9.1).

Screen house evaluation: Inoculum of each isolate was raised independently on millet grains. Pythium isolates were evaluated alone and in combination with the biocontrol agent. In one treatment the isolates were each mixed with pre-sterilized soil in a ratio of 1:8 v/v inoculum / soil in a wooden flat tray of 42 cm x 72 cm and left to stabilize for one week before antagonist MS10 was added in the same ratio and mixed with the soil. Planting was done after one week.

In another treatment inoculum of each of the five isolates (KB 14, KB 4, VIH 4, MS 61, and MS10) were each mixed with pre-sterilized soil in a ratio of 1:8 v/v inoculum to soil in wooden flat trays after which MS10 was added and planting done immediately. This was aimed at assessing the appropriate planting time after inoculation.

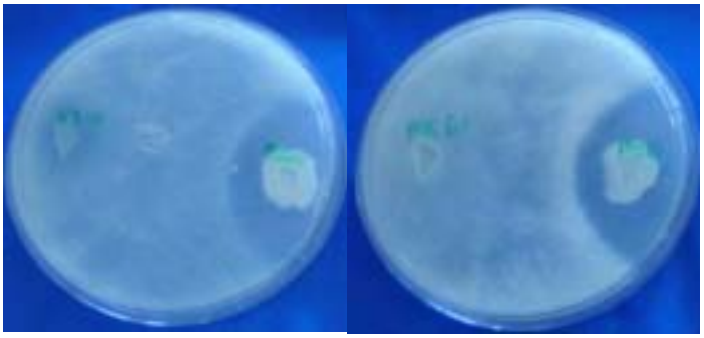

Figure 2.9.1. Level 3 of KB14 and MS61 vs MS10 on culture.

Twenty seeds of susceptible bean varieties CAL96 and K20 as well as resistant bean variety (RWR 719) were planted in two rows, each row consisting of 10 plants. A germination count was taken after full emergence. Disease evaluation was done 21 days after planting by uprooting the plants (Figures 2.9.2 & 2.9.3), rinsing the roots and scoring them according to the CIAT 1-9 scale where 1 = no visible symptoms, and 9 = complete discoloration of the tap root.

Figure 2.9.2. KB4 alone

Figure 2.9.3. KB4 vs MS10

Results and Discussion

Isolates KB14 (*P. ultimum*), KB4 (*P. vexans*) and MS61 (*P. ultimum* var. *ultimum*) applied singly showed severe root rot disease on CAL 96 confirming that these isolates are pathogenic to beans VIH4 and MS10 (*Mortierella*) isolates were non pathogenic to both susceptible bean varieties. Marked reduction in disease severity was observed in treatments where MS10 was added as antagonist to the pathogenic Pythium isolates KB14, KB4 and MS61. The highest disease severity was recorded in susceptible variety CAL 96 followed by K20. RWR 719 which is a resistant variety.

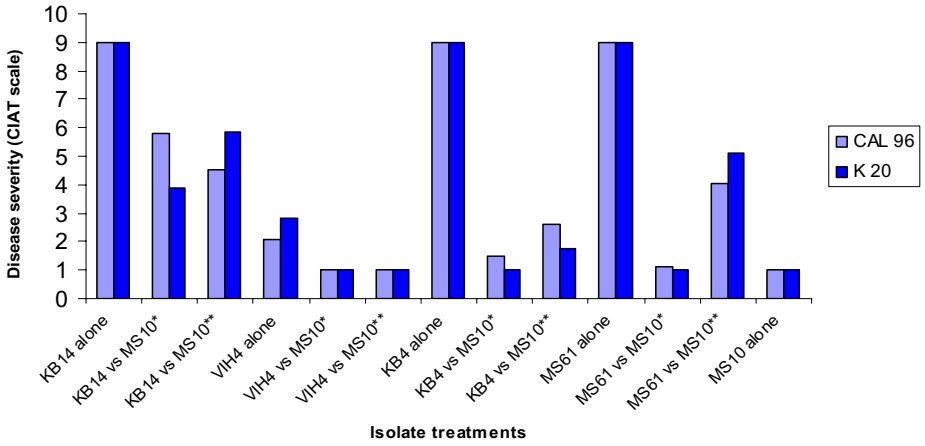

Figure 2.9.4. The effect of MS10 on pathogenic *Pythium* isolates: * = planting done one week after addition of antagonist MS10: ** = planting done immediately after addition of MS10

The results further indicate that application of MS10 one week prior to planting reduced disease severity better than applying followed by immediate planting. The one week interval before planting probably allowed the MS10 to colonize the soil and limit the effects of the pathogens. Application of MS10 also resulted in significant increase in root and shoot dry weights of susceptible varieties CAL 96 and K20 (Figures 2.9.4 & 2.9.5). It is logical to expect reduced root and shoot dry weight on plants with high disease severities as shown by isolate KB4 (highest disease severity and lowest root dry weight). It is not yet known how MS10 works but it was evident that its effects were influenced by the method and time of application. Microbial parasites and antagonists need time to have an effect.

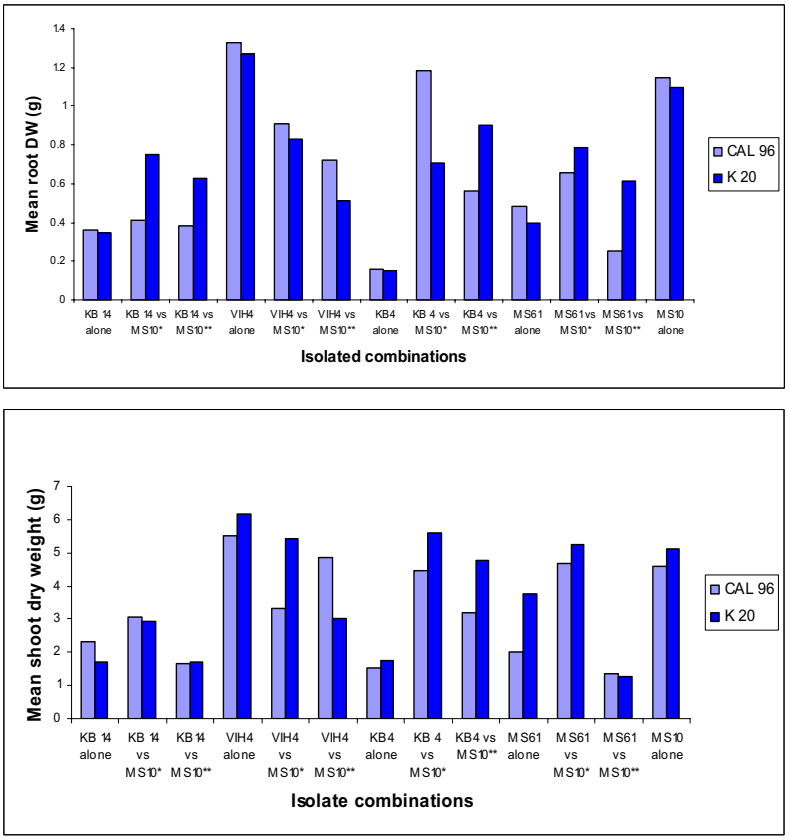

Figure 2.9.5. The effect of MS10 on mean root (top) and shoot (bottom) dry weight per plant of susceptible bean varieties CAL 96 and K20. * = Planting done one week after addition of antagonist MS10; ** = Planting done immediately after addition of MS10.

These results demonstrate the useful interactions mediated by MS10 in suppressing root rots and increasing root dry weight. However, the inoculum carrier used (millet grain, at a ratio of 1:8) may not be sustainable under field condition. These screenhouse results need to be verified under field conditions.

Activity 2.10. Farmers perception of Bean root rots and relationship to variety preference in Uganda

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Rationale

Despite beans being a major source of protein for most people in Uganda, there has been a great reduction in bean production. Bean root rots have been cited as one of the major cause of low bean yields in south western Uganda (CIAT, 1995, Strengthening collaborative bean research in sub-Saharan Africa, (PABRA), 61pp; Opio *et al*., 2001, Agriculture in Uganda, Vol. II CROPS: 162-191). Over time, this has resulted in a shift such that farmers have been forced to grow less preferred bean varieties due to the susceptibility of major preferred market class mainly the large seeded varieties. However, it has also been documented that large-seeded varieties lack resistance to Fusarium root rot (*Fusarium* f.sp. *phaseoli* - FSP). A study was therefore initiated to identify major bean varieties grown by farmers and their preferences; assess farmers perceptions on Fusarium root rot and its relationship to varietal preferences; the influence of root rot on varietal preferences; factors affecting bean yields that may or may not be related to Fusarium root rots the incidence and severity of Fusarium root rot in farmer's fields and farmers practices in managing root rots.

Materials and Methods

A participatory rural appraisal was carried out in two major bean producing and root rot affected areas in Uganda; i.e. south western Uganda; Kabale and Kisoro districts and eastern Uganda; Sironko and Mbale districts. Both interviews and focus group discussions were used to obtain both descriptive and numerical data. A questionnaire was designed, pre-tested and used to obtain information. Fifteen bean farmers per village were interviewed making a total of 120 farmers. The farmers interviewed were selected in a random and non-random manner (Systematic technique and accidental sampling). Data from the survey was analyzed using SPSS and Genstat computer programs. Focus group discussions were carried out in 2 villages per sub-county per district, with one group comprising of at least 15 people.

Results and Discussion

Farmer's perceptions of constraints to bean production: Farmers consider diseases, pests, excessive rainfall or too little rainfall, poor soil, soil erosion, lack of staking materials and drought as the major factors that constrain bean production and leading to the poor bean yields (Table 2.10.1). Diseases were the most mentioned factor in Kabale, Kisoro and Mbale while Sironko bean growers mentioned pests (aphids, bean stem maggot and storage pests) as the most important constraints. Too much rain was considered a constraint in Kabale, Kisoro and Sironko while drought was a major constraint in Mbale compared to other districts. This is because generally, Mbale receives less rainfall compared to other districts. Soil erosion was considered a problem in Kabale and Kisoro where beans are grown on mountain slopes due to lack of enough land. In addition, lack of staking material is a problem in southwestern Uganda, where climbing beans are popular.

It was clear that root rot is considered the most important disease of beans in south western Uganda and in Sironko, while not being as important in Mbale. This is likely to be related to the amount of rain received in each region. Other constraints mentioned included aphids, burning like disease, rats and bird. It was apparent that farmers could not differentiate between diseases and pests.

Table 2.10.1. Farmer's perceptions of constraints to bean production.

Constraint	Kabale	Kisoro	Mbale	Sironko
Diseases	93.3	95.0	80	73.3
Pests	30	24.6	77.1	93.3
Excessive	66.7	62.5	22.9	63.3
Poor soil	16.7	22.1	2.9	6.7
Soil erosion	36.7	26.7	0	0
Lack of staking material	26.7	30.5	0	0
Drought/a lot of sunshine	30	25.5	74	8.3

A pair wise ranking (Table 2.10.2) done in Kisoro clearly indicated that root rot was the most important biotic constraints, followed by burning and finally aphids. Rats are not considered very important to bean production though they cause some damage. Other constraints were birds and bean stem maggot. Bean root rots were ranked highest in south western Uganda and Sironko while in Mbale drought was ranked as the major constraint.

Table 2.10.2. A pair wise ranking of farmer's perceptions of bean diseases and pest in Kisoro, Nyarusiza, Village.

	Root rot	Rats and moles	Burning	Cut worm	Bean fly
Rats and moles	Root rot				
Burning	Root rot	Burning			
Cutworm	Root rot	Rats	Burning		
Bean fly	Root rot	Rats	Burning	Cut worm	
Aphids	Root rot	Aphids	Aphids	Aphids	Aphids

Root rot= 5, Aphids=4, Burning= 3, Rats=2, Cutworm=1

Farmer's perceptions on Fusarium root rot: Generally farmers in Kabale and Kisoro (100% of the farmers interviewed) are aware of bean root rots and are aware of its devastating effects on bean yields. In Kabale it is referred to as "*Kiniga*" (Rukiga) and in Kisoro as "*Kirusuka*" (Rufumbira). It is ranked as the highest cause of bean yield losses followed by other factors as shown below. Similarly in Mbale and Sironko farmers did recognize root rot as a disease of beans, i.e., 85.7% in Mbale, and 86.7% in Sironko. In eastern Uganda, the Bagisu refer to it as "*Ukwishikula*", "*Washa*" or "*Kyengu*" depending on what symptoms are seen. However, it is not as an important constraint to bean production as compared to Kabale and Kisoro, i.e., 37.1% in Mbale and 70% in Sironko considered root rot important compared to 93.3% of the farmers in Kabale (Figure 2.10.1).

Causes of root rots: Farmers associate the occurrence and severity of bean root rot to a number of factors including, poor soils, shallow soils caused by soil erosion since most fields are on hill slopes, drought, over cultivation of soil caused by land fragmentation, mist (this settles on plants in the mornings and evenings). Others associate bean root rot to traditional beliefs and witch craft as whole bean fields are lost in some seasons. Cultural rituals are practiced in some homes in case of a root rot epidemic to control it.

Other factors said to be predisposing beans to root rot were; insects in the soil, pests such as bean stem maggot, drought/a lot of sunshine, lack of crop rotation, planting under trees, intercropping especially with maize, lack of resistant varieties and standing water/ stagnation.

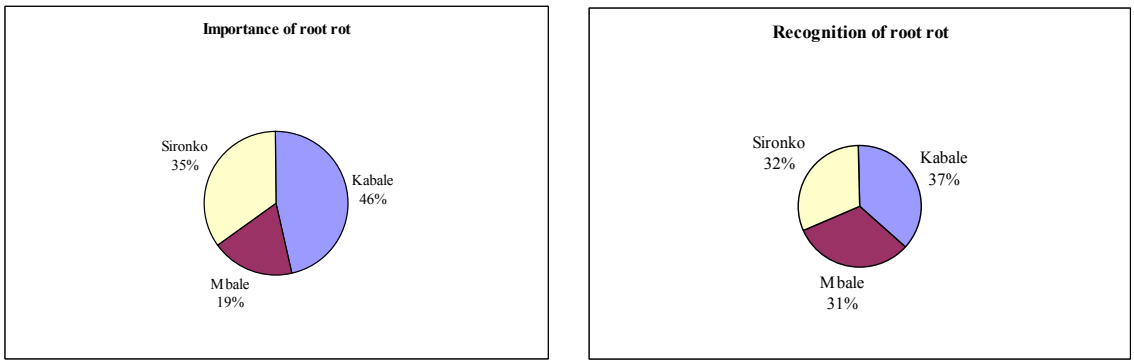

Figure 2.10.1. Recognition and importance of Root rots in Mbale, Sironko and Kabale.

Farmers practices to manage bean root rot: A variety of practices are used in managing root rots and include; addition of fertilizer, spraying with chemicals, timely planting, good bean variety (resistant varieties), good quality seed, soil conservation using drainage trenches, resting of soil, manuring, crop rotation, intercropping planting improved varieties, fallowing, ash, weeding, traditional methods, hilling up, planting mature seed, rouging and burying infected plants, spraying or nothing at all (Figure 2.10.2). The majority of farmers; 49% do nothing to control root rots, while a few practice manuring (mainly in south western Uganda) and crop rotation.

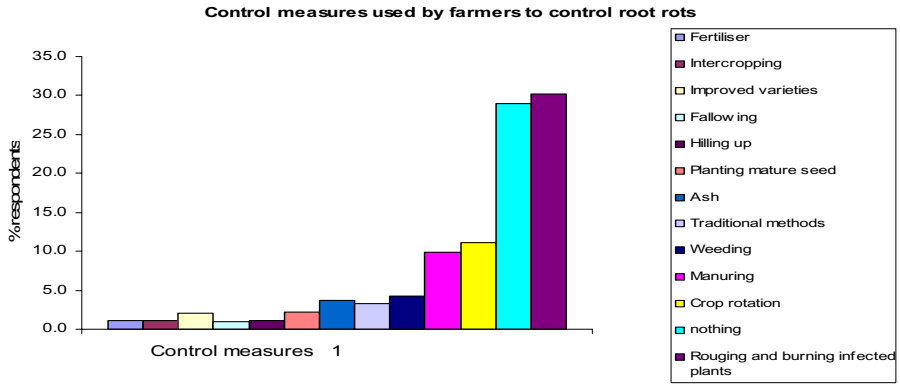

Figure 2.10.2. Management practices used by farmers to manage root rots.

Farmer's perceptions of relationship between Fusarium root rot and variety preference: Bean growers consider taste, marketability, yield, seed size (majority prefer large seeded varieties), seed appearance, soup color/ seed color when considering taking up a new variety. Some farmers have not made any observations on the effect of a variety on the occurrence and severity of root rots but a few especially in

the south western associate the large seeded beans to being susceptible to root rots. A few have observed that small seeded, which are less preferred are generally less affected by diseases including root rots.

Activity 2.11. Developing germplasm with multiple resistance to viral diseases

Contributor: Francisco Morales

A. Bean common mosaic/necrosis viruses

All advanced lines produced by CIAT are expected to possess resistance to bean common mosaic and bean common mosaic necrosis viruses. This activity includes lines improved for their resistance to abiotic stress, such as drought. This year, 305 drought materials were evaluated for their reaction to *Bean common mosaic virus* (BCMV) and *Bean common mosaic necrosis virus* (BCMNV). The first batch of 189 materials showed the presence of recessive genes, probably *bc-3* or protected *I* gene that confer resistance to all virus strains, in approximately 66% of the materials evaluated. Approximately 7% of the materials possessed monogenic dominant resistance (susceptible to necrosis), and only 3.2% of the materials was susceptible to mosaic. In a second trial, 234 materials selected for drought tolerance, architecture and Iron content, the number of lines possessing monogenic dominant resistance to common mosaic increased to 97%.

The incorporation of broad-spectrum recessive resistance to BCMV and BCMNV has been increasingly important in the Bean Project, specially in past years. This type of resistance is stable and prevents undesirable linkage problems associated with certain commercial seed colors and susceptibility to these viruses. This year, a total of 3,012 materials were evaluated for the presence of the responsible gene, *bc*-3. In these inoculations, 20% of the test materials proved immune to mosaic and necrosis induced by BCMNV NL3, the most pathogenic of the BCMV and BCMNV strains found in nature. Over 7% of the materials inoculated had monogenic (*I*) resistance, and 45% were susceptible to mosaic. Approximately 20% of the materials inoculated proved to be segregating for the *bc-3* recessive gene.

One of the most commercially demanded and valuable climbing common bean cultivars in Colombia, is 'Cargamanto'. Unfortunately, this cultivar has been under attack from BCMV in the highlands of Antioquia, the main production area. 'Cargamanto' also presents linkage problems between its commercial seed coat characteristics and susceptibility to the virus, associated with monogenic dominant resistance conferred by the necrosis *I* gene. Consequently, the incorporation of broad-spectrum recessive resistance to BCMV has been the main strategy to improve this valuable variety. This year, we continued the screening of Cargamanto lines produced by CORPOICA. Of a total of 94 materials evaluated this year, 13% proved to be homozygous immune. The *bc-3* gene was present in 58.5% of the materials found to be segregating for this gene. 28% of the materials were devoid of any dominant or specific recessive resistance genes against BCMV/BCMNV.

Additionally, Bean Virology screened 281 parental materials for breeding purposes. 16% of these parental materials were immune to BCMNV-NL3, which indicates the presence of recessive genes, such as *bc-3*, in these parental genotypes. The majority of these genotypes, however, have the necrosis *I* gene. Only 3.5% expressed common mosaic, and the rest of the materials were segregating for all genes. A total of 227 breeding materials were also evaluated for their reaction to BCMV/BCMNV. About 29 lines for the Middle East were totally susceptible (mosaic); whereas 90 RAB and MAB lines showed monogenic dominant resistance to BCMV.

B. Bean golden yellow mosaic virus

Bean golden yellow mosaic virus (BGYMV) is still the number one biotic constraint to bean production in Mesoamerica, including the Caribbean Region. So far, 0ver 25 years of research at CIAT and collaborating national programs has paid off in terms of BGYMV-resistant common bean varieties released, and sources of resistance made available to national and international institutions in this region. International programs, such as USAID's CRSP/TXII and the Panamerican School in Honduras, have been releasing new BGYMV-resistant bean lines based on the available sources of virus resistance.

At CIAT, the BGYMV work continues but it is subordinated to the incorporation of abiotic traits, namely drought tolerance and iron (Fe) content for Central America. In 2005, 407 materials were evaluated for their reaction to BGYMV. A high proportion of F7 genotypes (70%) had an adequate level of BGYMV resistance; as well as 18.5% F8 genotypes from mass selections. The rest of the materials segregated resistant and susceptible. A mass selection of F8 materials for high Fe content, yielded 28 resistant lines (R) out of 71 materials evaluated for their reaction to BGYMV; 9 lines were susceptible (S); and 34 lines were segregating R&S.

The number of resistant materials belonging to different breeding projects were: 8/49 (RCB and NCB); 5/29 (F6 archit/Fe); 5/19 (MIB); 4/28 (MAB-A); and 36/51 (MAB-B).

C. Bean leaf crumple virus

This is the new whitefly-transmitted begomovirus that has practically eradicated snapbean production from the flat lands of the Cauca Valley (*c.* 1000 m above sea level). This virus appears to be a hybrid between the Mesoamerican BGYMV and other begomoviruses that have been introduced in past years in the Cauca Valley, and are currently affecting unrelated crops, such as tomato. In past annual reports, we have reported that Bean Virology, in cooperation with Bean Entomology and Breeding, has identified sources of resistance to this virus in Mesoamerican black-seeded common bean genotypes (Figure 2.11.1).

Although the new biotype (B) of the whitefly species *Bemisia tabaci* is responsible for the outbreaks of this new virus in snap bean plantings in the Cauca Valley of Colombia, the original biotype (A) is still a more efficient vector of this virus. An average disease incidence of 4.9% was obtained for biotype B in six different biological transmission tests, as compared to a 26.6% incidence for the A biotype, using only one viruliferous whitefly per plant. In these tests, the black-seeded parental materials Porrillo Sintetico and BAT 304, registered the lowest disease incidence (0 and 7%, respectively) using up to 25 whitefly individuals per plant. Topcrop and Red Kloud showed incidence of 100% in these tests. A black-seeded commercial cultivar like ICTA-Ligero, suffered a 27% virus incidence.

Resistance to bean viruses, such as BCMV, BCMNV and BGYMV is readily available and highly resistant common bean varieties have been developed from these sources for Central America. In Honduras and El Salvador, two red-seeded common bean varieties have been released in the past year, with excellent adoption thanks to their commercial seed color (Rojo de Seda) and good cooking characteristics. In El Salvador, these varieties are called CENTA-San Andrés (Figure 2.11.2) and CENTA-Pipil, both bred by Dr. Juan Carlos Rosas of El Zamorano, Honduras. In a recent trip to El Salvador, several lines possessing recessive (*bc-3*) resistance to BCMV and BCMNV, were also showing resistance to the main virus problem of Central America, BGYMV. This is a major accomplishment in a region that requires both types of virus resistance without compromising grain quality.

Figure 2.11.1. Broad pathogenicity range of a new whitefly-borne virus in common bean genotypes under field conditions in the Cauca Valle, Colombia.

Figure 2.11.2. Bean golden yellow mosaic-affected cultivar and resistant cultivar developed by El Zamorano, Honduras

Activity 2.12. Efficiency of entomoparasitic nematodes as biopesticides versus *Phyllophaga menetriesi* and *Anomala inconstans* in relation to host age

Contributor: E. L., Melo; C. A. Ortega, A. Gaigl

Rationale

The genera *Phyllophaga*, *Astaena*, *Anomala*, *Isonychus*, and *Macrodactylus* are the economically most important white grubs of the family Melolonthidae in Colombia (more than 600 species) Chemical control of these pests is not feasible due to its costs, environmental risks and the lack of knowledge in fundamental aspects such as population dynamics complicating the development t of control strategies (Falcon & Smith, 1983, El concepto de control integrado de las plagas, UNDP and CIAT, pp 15-20).

The microbial control of white grubs is widely considered as a valuable strategy of a sustainable pest management. Most studies on biological control focus on the third instar larvae (Jackson & Brooks, 1995, J. Nematol 21: 15-20) that cause more damage than those in earlier instars (King, 1984, Tropical Pest Management 30: 36-50.). However, many works with entomoparasitic nematodes (EPN) resented difficulties in controlling this instar (Koppenhöfer & Fuzy, 2003, Biological Control 28: 47 – 59; Simard *et al.*, 2001, Suppl. Journal of Nematology 33: 297-301).

The objective of the present work is to evaluate native strains of EPN on two white grub species at two different stages of development.

Materials and Methods

We conducted this experiment in the laboratory of Cassava Entomology at CIAT, under controlled conditions (25.4ºC and 86% RH) at complete darkness. We tested the two native EPN strains *Steinernema feltiae* (Villapinzón, Cundinamarca) and *Heterorhabditis* sp. (Fresno, Tolima) at a concentration of 10,000 Dauer Juveniles (DJ) per milliliter.

The white grubs (*Anomala inconstans* and *Phyllophaga menetriesi*) were taken from the first generation of CIAT's white grub colony (23 ºC, 70% HR). The first instar larvae were confined in a plastic vessel of 5 liters. The other instars were confined in plastic cups filled with 70 g soil (sand : soil organic matter = 1:1) and fed on roots of young rice plants. We used first, second, and third instar larvae, latter at two and four weeks after molting according to the availability of the different ages of each species during November 2004 and March 2005

We tested both EPN species on *A. inconstans* whereas we applied only *Heterorhabditis* sp. on *P. menetriesi* because *S. feltiae* failed to control larvae in the first instar.

The experimental unit was defined by 12 plastic cups (21 g) filled with humid soil (field capacity). Each cup harbored one white grub. The checked the larvae on mortality after 10 and 20 days after application (daa) of the nematodes. Since the data were not normally distributed after applying Abbott's formula (1925) we transformed them by means of the square root $\sqrt{x\,2\,1}$ prior to ANOVA. Normal distribution of the transformed data was corroborated by using the Shapiro–Wilks Normality Test (1965). The differences of means were tested on significance by the Tukey–Test (PÒ0.05) (Infostat 2004, Grupo INFOSTAT, FCA., Universidad Nacional de Córdoba, p318).

Results and Discussion

Survival of white grubs in the control treatment depended on the age. L1 showed with 16% the highest mortality whereas three percent larvae of L2 died without being effected by any pathogen . Interestingly, more larvae as young L3 instar died than as L2; however, differences were not significant. All advanced L3 instar and pupae survived in the control treatment. In general mortality of untreated white grubs was 11%.

Larvae 2 (L2) of *A. inconstans* was the most susceptible instar to *H. bacteriophora* whereas larvae 3 (L3) was the less affected stage by *S. feltiae*. *H. bacteriophora* was more pathogenic to all instars than *S. feltiae* and we didn't find any differences between the two evaluation times (10 and 20 dda).

The second instar of *P. menetriesi* was the most susceptible stage to EPN. This susceptibility was reduced with increasing age of the insect. We observed differences between evaluation times: mortality was significantly greater after 20 dda. According to these results we suggest to apply EPN as biopesticide when larvae of *P. menetriesi* are in the second instar.

Comparing the two white grub species we observed that *A. inconstans* is more susceptible to an EPN attack than *P. menetriesi*, our results corroborate earlier studies where only 10% of L3 were killed by EPN (CIAT, PE-1 Annual Report 2004). Both soil pests presented highest mortality in the stage of L2

Several reasons may explain the variation in susceptibility between stages of development: (i) immune response associated with the age; (ii) size and behavior of the host; (iii) the smaller diameter of the spiracles of young larvae (Jackson & Brooks, 1995, J. Nematol 21: 15-20); (iv) reduced production of CO_2 and less kairomones of L1 and L2 (Kaya,1985, J. Invertebr. Pathol. 46: 58-62); (v) thickness of cuticle of L3 increases the difficulties of penetration (Koppenhöfer & Fuzy, 2004, Ento.Soc.of America. 97:1842-1849); (vi) the fact that larvae reduce and stop feeding activities when they are approaching the pre-pupal phase lowers the possibility of being penetrated by the nematode via the mouth. Clearly, the developmental stage of the host insect has an important impact on the efficiency of EPN. Moreover, the extent of this effect depends on the species of host and nematode (Koppenhöfer & Fuzy, 2004, Ento. Soc.of America. 97:1842-1849)

Conclusions

- *A. inconstans* presents greatest mortality when *Heterorhabditis* sp. penetrates the second instar (98.3%).
- The most susceptible stage of *P. menetriesi* is the second instar infected by the species *Heterorhabditis* sp. (mortality of 81.1%).
- *P. menetriesi* is more resistant to EPN than *A. inconstans*. Both white grub species are most susceptible during the phase of second instar.
- All developmental stages of *A. inconstans* didn't show any mortality in terms of natural mortality. In contrast, the first instar of *P. menetriesi* showed the greatest mortality, followed by the second. Latter observation is expected since younger organisms are more susceptible to parasitic organisms due to the morphological, physiological, and behavioral characteristics.
- We recommend applying entomoparasitic nematodes as biopesticide versus the second instar of white grubs.

Activity 2.13. Evaluation of different concentrations of the entomoparasitic nematode *Heterorhabditis bacteriophora* (Italia) versus second instar larvae of *Phyllophaga menetriesi* (Coleoptera: Melolonthidae)

Contributors: Elsa Liliana Melo, Carlos Alberto Ortega, Andreas Gaigl

Highlight:

∉ New promising natural enemy of *Phyllophaga menetriesi* identified

Rationale

The larvae of scarabs (Coleoptera: Melolonthidae) are considered as some of the most rhizophagous pests on a wide range of crops such as pasture, cassava, maize or ornamental flowers and many others. *Phyllophaga menetriesi* is the most aggressive and noxious soil pest in the climatic range between 1000 and 1600 m.a.s.l. (CIAT, Annual Report 2004, Integrated Pest and Disease Management in Major Agroecosystems, 417 p). For this reason this species was object for experiments on the efficiency of entomoparasitic nematodes (or entomonematodes) as agents of biological control.

The biological control by nematodes was already suggested in the 1930's (Jackson, 1993, Diversidad y Manejo de Plagas Subterráneas, Soc. Mex. Ent, 261 pp). Bacteria, fungi, virus, and entomonematodes have been used until the present for programs of biological control; however, the use of them against scarabs is limited due to the absence of proper filters, methods of mass production, and inappropriate application. The most successful programs of microbial biocontrol are based on pathogens that are highly adapted to a well defined range of pests. Even the age of the target organism has an important impact on the efficiency of biological control agents such as entomoparasitic nematodes (Melo *et al*., 2005, Resúmenes del XXXII Congreso de SOCOLEN, Ibague, Colombia, p 80).

In the present study we wanted to analyze the potential of the introduced entomoparasitic nematode *Heterorhabditis bacteriophora* as bioinsecticide against the second instar of *Phyllophaga menetriesi* under controlled conditions applying in different concentrations.

Materials and Methods

We conducted this experiment in the laboratory (23 ± 2ºC, R.H. 70±5%, and complete darkness.

Target Insect: We received larvae of *Phyllophaga menetriesi* in the second instar from our lab colony. The larvae were confined in transparent plastic cups of 150 ml with lid. The cups were filled with organic soil (one part organic matter and two parts sand). The white grubs fed on young rice plants (grubs were introduced when plants passed 20 days after germination.

Entomoparasitic nematodes: We used the Italian strain CTN003 of the nematode *H. bacteriophora* that was one of the most pathogenic strains in previous testes (CIAT, Annual Report 2004, Integrated Pest and Disease Management in Major Agroecosystems, 417 p). Nematodes were applied in seven doses: 32, 100, 307, 960, 2000, and 3000 Dauer Juveniles (DJ) per milliliter. These numbers were obtained using arbitrary scale of $\log_{10}$. We applied distilled water as control (no nematodes). We evaluated the experiment 20 days after application (daa) counting the killed larvae. The dead insects were transferred to a White trap (White, 1927, Science 66: 302-303) in order to verify and recover the nematodes.

Experimental Design: The experimental design was at random. All treatments were repeated four times. Each experimental unit harbored 13 larvae. Prior to ANOVA data were transformed by using Abbott's formula (1925) and then by the square root $\sqrt{x\,2\,1}$. Normal distribution of the transformed data was corroborated by using the Shapiro–Wilks Normality Test (1965). The Tukey-Test (PÒ0.05) was used to analyze statistical differences of means (Infostat 2004, Grupo INFOSTAT, FCA., Universidad Nacional de Córdoba, p318).

Results and Discussion

A relative low concentration of only 307 DJ/ml caused a mortality of more than 80%, a concentration of 960 DJ/ml killed more than 90% of the grubs (Figure 2.13.1) the best result of all applied concentrations; more DJ/ml maintained this level of mortality. This indicates that this strain can be applied at low concentrations. This mortality is striking compared to the results obtained applying nematodes on the third instar where 10,000 DJ/ml obtained mortality of only 12% (CIAT, Annual Report 2004, Integrated Pest and Disease Management in Major Agroecosystems, 417 p).

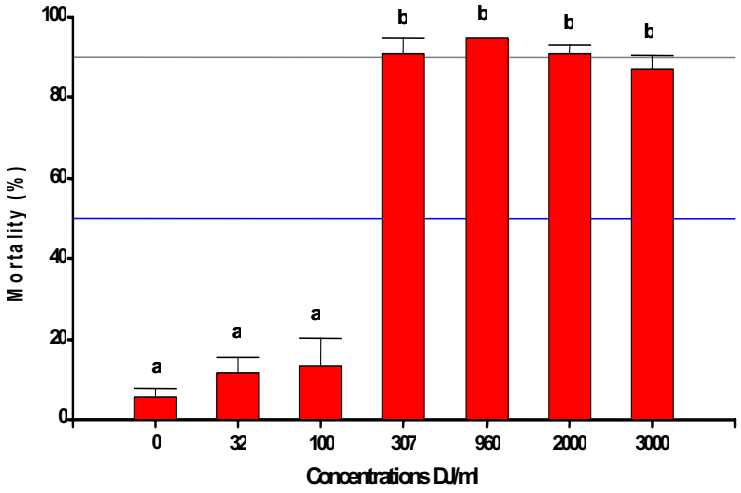

Figure 2.13.1. Mortality of *Phyllophaga menetriesi* (larvae in second instar) in relation to different concentrations of Dauer Juveniles (DJ) of *Heterorhabditis bacteriophora* (Italian strain CTN003).

P. menetriesi is a white grub species that presents a strong resistance to nematodes (Koppenhöfer & Fuzy, 2004, Entomological Soc. of America 97: 1842-1849 ; Simard *et al*., 2001, Suppl. J. of Nematology 33: 297-301) that is indeed an important limitation for the use of entomopathogenic organisms (Cappaert & Koppenhöfer, 2003, Biological Control 28: 379– 386). However, in previous experiments other EPN strains such as *Heterorhabditis* sp., *S. carpocapsae*, *S. glaseri*, and *S. longicaudum* showed promising results versus the second instar of *Anomala orientalis* (Lee *et al.,* 2002, J. of Economic Entomology 95: 918 – 926). These data show that the probability of a successful control is much better when the second larval instar is targeted (Melo *et al*., 2005, Resúmenes del XXXII Congreso de SOCOLEN, Ibague, Colombia, 80 p) suggesting including native strains in the tests of biological control determining the most efficient concentration that are commercially feasible.

When the phenology of the pest insect is known the best time of applying this bioinsecticide can be easily defined, for example *P. menetriesi* is present in Northern Cauca during the months November and

December. Moreover, we suggest conducting experiments combining nematodes with other microbial control organisms and other IPM strategies.

Koppenhöfer & Fuzy (2004, Entomological Soc. of America. 97: 1842-1849), suggest that the efficiency of the EPN depends on the host's age; however, this effect depends on the host species and the applied EPN. Moreover, we find that the commercial dose of 500 DJ/ml indicated by "E-nema" for the hybrid species *H. bacteriophora* is even greater than the optimal concentration of the Italian strain.

Although our Italian strain of *H. bacteriophora* showed highly promising results we recommend for future research continuing the search for native entomoparasitic nematodes due to two reasons: Firstly, in this experiment we couldn't include more nematodes for direct comparison and secondly, the release of exotic organisms in the field may raise legal conflicts due to the national policy of environmental protection.

Knowing that the second instar the larvae of *P. menetriesi* is the most susceptible stage to entomoparasitic nematodes (Melo *et al*., 2005, Resúmenes del XXXII Congreso de SOCOLEN, Ibague, Colombia, 80 p) we suggest continuing research defining best concentration for other strains of nematodes. Our results also help to determine the optimal moment for application of this bioinsecticide according to the pest's phenology.

Many aspects remain for further evaluation in order to respond to farmers' inquiry how and when to control this pest. Since the studies on entomopathogenic organisms against *P. menetriesi* are still in the beginnings this would be the first step for a sustainable biological control of this pest species.

Activity 2.14. Lethal density of *Phyllophaga menetriesi* (Coleoptera: Melolonthidae) associated with maize, beans and cassava plants

Contributors: A. Ortega-Ojeda, E. L. Melo-Molina, A. Gaigl

Highlights:

- ∉ Action threshold of white grub species *Phyllophaga menetriesi* on three crops defined
- ∉ Agricultural methods as strategies of integrated pest management described

Rationale

White grubs (Coleoptera: Melolonthidae) belong globally to the soil biota. The major part of them plays an important role in the recycling of soil organic matter; however, some of them are important pests on a wide range of crops. Especially in the tropics the diversity of this order is impressing. In Colombia more than 600 species of the family Melolonthidae are known. In Pescador Caldono (Cauca) we know 44 species; some of them are of major economic importance such as *Phyllophaga menetriesi*, *Phyllophaga* spp., *Plectris fassli*, *Pl. pavida*, *Ceraspis innotata*, *Astaena valida*, *Anomala inconstans* and *A. cincta* (Pardo, 2002, MSc Thesis, Universidad del Valle, Cali, Colombia, p 33-82). All of these species *P. menetriesi* is the most voracious one on a wide range of tropical crops. The most aggressive phase is the third larval instar and with a length of four centimeters is one of the biggest rhizophagous white grubs (*ibid*.). The third instar is not unique only in terms of aggressive behavior with tendencies to cannibalism but also in terms of resistance to synthetic and biological insecticides (CIAT, PE-1 Annual Reports 2003 and 2004)

White grubs attack crops such as maize or beans at the succulent parts below the soil surface feeding on roots and germs. Damage on cassava is characterized by damage both of bark and internal cambium as well as perforation and tunnels in swollen tubers.

Farmers often do not understand the relation between damage and pest due to its cryptic habitat. Frequently they replace in vain lost plants with new seed only to continue alimenting the grubs and increasing production costs. At present there exist only a few reports on the threshold for white grubs that state that three larvae associated with maize or cassava and coffee, respectively, are necessary to destroy the host. Farmers often see in the use of synthetic insecticides the only possible strategy to minimize losses. Mostly, this strategy only increases the production costs and threatens farmers' and environmental health. Moreover, the development of resistance in these insects is another secondary result.

The objective of this experiment was to establish a threshold of white grub density and develop a tool that helps farmers in decision making when they have to apply control activities against this pest associated with maize, cassava, and beans.

Materials and Methods

This experiment was conducted on the farm "Bellavista" in Pescador (Cauca, Colombia) at an altitude of 1580 m.a.s.l. We selected as host plants maize (variety ICA-305, hard endosperm), red beans (variety "Toné"), and stems of cassava (variety SM 707-17). 15 days old plantlets of maize and beans and the cassava stems were placed individually in plastic buckets (height: 40 cm, diameter: 30 cm, volume: 28,000 cm^3) filled with soil taken from the farm. At the bottom the buckets had drainage of a 6 cm diameter. The treatments consisted of five white grub densities (0, 1, 3, 5, and 7) per bucket. Every treatment was repeated six times.

We chose a randomized complete block design (CRD) for a two-factors experiment where beans and maize were the host plants. The two factors were three evaluation periods (nine days for each period) and the white grub densities. In the case of cassava we only considered the factor white grub densities due to the long growing time of this crop.

Only larvae in the third instar of *P. menetriesi* were used. The insects were taken from the rearing colony at CIAT and confined in the buckets according to the defined densities. The larvae that did not dig into the soil within a lapse of ten minutes were replaced.

We tried to simulate natural conditions for the white grubs as good as possible. We inserted the buckets by 95% of their height into the soil in order to level the experimental unit with the surrounding field and to avoid direct sunshine on the borders and avoid fluctuation of temperature. In each of these buckets we planted one cassava stem or a 15 days old maize or bean plant. One day later we released larvae in the third instar according to the assigned density. When beans and maize were the hosts we started the daily evaluations one day later. These observations lasted nine days and were repeated three times over time. New plants were inserted when a new repetition over time started; however, the grubs remained in the buckets. When cassava was the host we started evaluations after 17 days after planting the stem and collected data of the experiment every fourth days during a lapse of 60 days. During this period we observed plant mortality, estimated plant vigor, development, and color of the foliage. The scale for the estimated plant vigor levels ranged from 1 to 9, where 1 corresponded to a plant in ideal shape and 9 to irrecoverable. We adopted this scale from Schoonhoven & Pastor-Corrales, 1987, CIAT who applied this methodology for evaluating germplasm of beans. Once a plant was dead we extracted the stake and analyzed internal and external damage due to white grub attacks.

Before we run the statistical analysis we checked the data on normal distribution by means of the Shapiro-Wilks-Test (1965). Since the data from the experiment with cassava showed an elevated variation and values of zero we transformed the data applying the formula $\sqrt{x\,2\,1}$. Data were analyzed by means of two-ways ANOVA (beans and maize) and one-way ANOVA (cassava) using software INFOSTAT (2005) to determine single or interaction effects of factors. In case of significant F-Values we compared means using Tukey's test.

Results and Discussion

Maize: The plants of the control treatment reached a height of 23.32 cm nine days after infestation (dai). All plants of the other treatments died before this period. Plants associated with one grub showed the longest agony (3.5 dai) reaching a height of 17.55 cm. In contrast, plants associated with five or more grubs died within 2.4 dai (Figure 2.14.1a) (pÒ0.0007). According to these results we ruled out the conclusions by Ayala & Monterroso, 1998, Manual para Técnicos 2, Costa Rica, p. 7-8 and Pardo *et al.,* 2003 (In: Estudios sobre Coleópteros del suelo en América, Aragon *et al*. (eds), U. Autónoma de Puebla: 283-297) that at least three larvae of *P. menetriesi* are necessary to destroy one maize plant. We recommend not tolerating losses more than 5% of 50 randomly selected plants per hectare.

The white grubs eliminated the plants faster in the first repetition (2.2 dai) than in the other two (2.8 dai) (Figure 2.14.1b) (pÒ0.05). We conclude that the larvae of *P. menetriesi* reduce feeding activities while approaching the end of the third instar giving the farmer the chance to escape the white grub attack by sawing the crops when the larvae are older or have completed the larval stage

Beans: Only the plants in the control treatment survived this experiment. They reached a height of 27.92 cm after 5.2 days when the last plant of treatment 3 (one grub per plant) died having reached an altitude of 24.13 cm. Three and more larvae needed 3.1 days for destroying one plant (Figure 2.14.2a). This corroborates Arguello *et al.* (1999, PROMIPAC-Nicaragua, 18 p), who observed that white grubs frequently destroy 100% of a plot within seven or ten days. Likewise as in the case of maize we conclude that the threshold for beans is only one larva per plant and recommend not permitting plant losses greater than 5%.

In the first repetition larvae of *P. menetriesi* needed 3.21 days for elimination of the plants and in the third 4.5 days (Figure 2.14.2b). This confirms our observations in earlier field and greenhouse experiments that this white grub species reduces its feeding activity when it completes the larval stage. As in the section before we recommend to elude the presence of *P. menetriesi* by altering the date of planting.

Cassava: Only one larva is able to cause an external damage of 53% of the stake feeding on bark and 6.7% on the internal cambium (Figure 2.14.3). Five or seven grubs eliminate almost completely the bark of a stem and eliminate approximately 30% of the cambium. Three larvae reduce the bark by 80%. Damage 24 dai was clearly proportional to white grub density (pÒ0.05) (Figures 2.14. 4 and 2.14.5).

After 49 days one grub damaged a plant by 30% (Figure 2.14. 6). Observing the vigor of the plant and according to earlier observations (Ortega *et al*., 2005, Memorias XXXII Congreso SOCOLEN, Ibague, Colombia, 89 p) we conclude that the attacked plants may continue their development; however, their yield can be heavily reduced.

These results confirm that it is possible to identify levels of damage estimating the vigor of the plant. However, these indicators have to be detected very of the crop because the threshold for cassava is below one white grub per plant. If the visible plant damage is below level 3 (= slight damage) it is possible to keep yield reduction below a range 5%.

We concluded that:

- ∉ One white grub destroyed a maize plant within 3.3 days; three or more larva needed 2.4 days.
- ∉ One larva needed 5.2 days to destroy the one bean plant; three or more larvae needed 3.1 days.
- ∉ The probability was 70% that a cassava stake survives the attack of one white grub; however, we estimated that this damage could reduce yield by 53%.
- ∉ The probability that three white grubs eliminate completely one cassava stem in 56 days is 50%. Seven grubs reduced this period down to 24 days. The probability that seven grubs destroy one cassava stem within 35 days was 100%.
- ∉ The threshold for cassava, beans, and maize is below one white grub associated with a plant.
- ∉ We recommend varying the date of sawing beans or maize in order to elude the presence of the larval instar III.
- ∉ In order to keep plant losses in maize and beans below 5% we recommend revising randomly selected 50 plants per hectare. When three plants of those are associated with three white grubs farmers should get active against this pest.

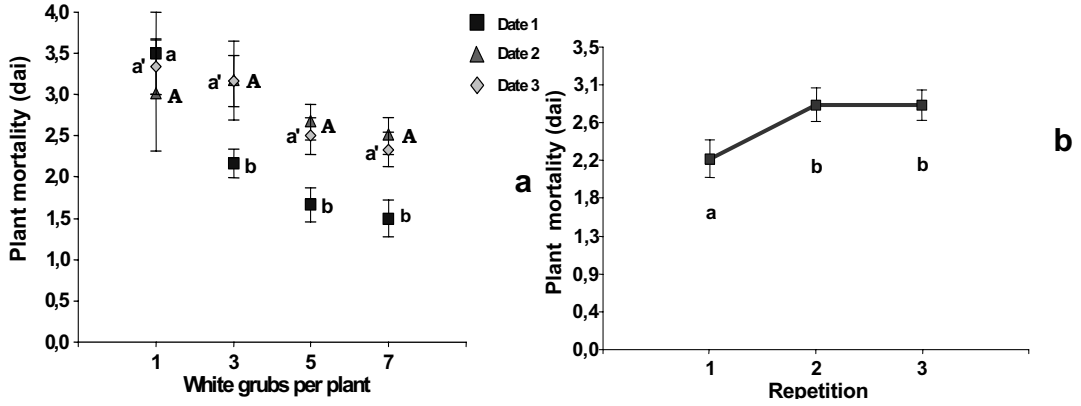

Figure 2.14.1. Mortality of maize plants. **a**) mortality in each repetition **b**) means of each repetition (Tukey pÒ0.05).

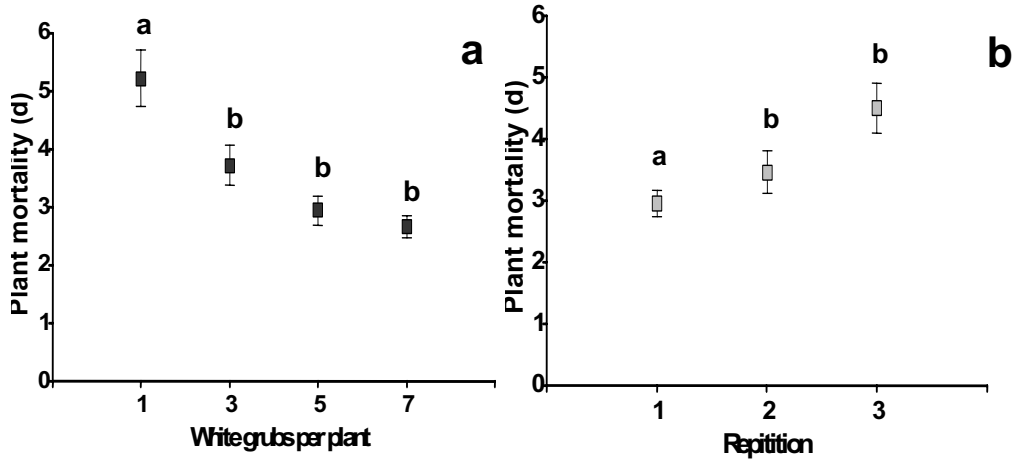

Figure 2.14.2. Mortalityof bean plants. **a**) means of treatments per repetition; **b**) means per treatments; y, c. all means per repetition (Tukey pÒ0.05).

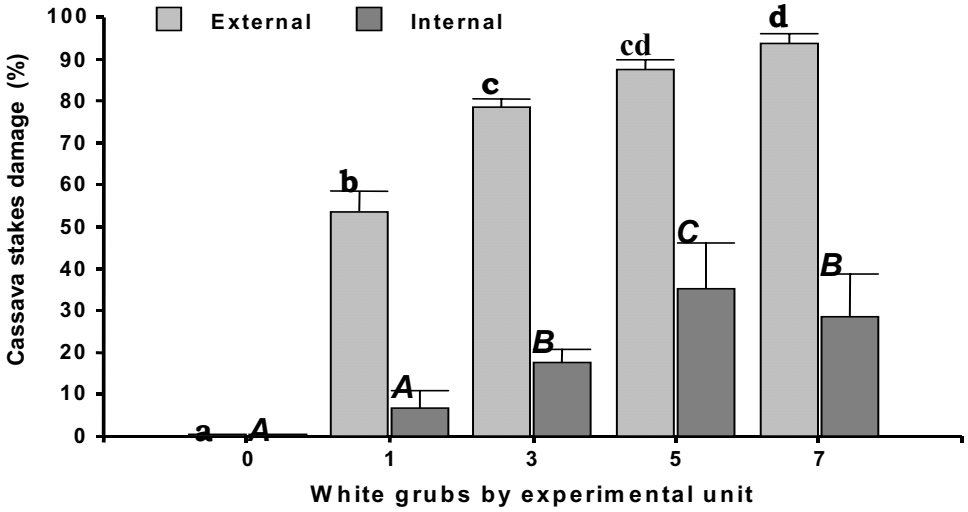

Figure 2.14.3. External and internal feeding damage on cassava stakes in relation to 0, 1, 3, 5 y 7 larvae (Tukey pÒ0.05).

Figure 2.14.4. Differences in vigor (left) and external and internal damage (right) due to white grub attacks on cassava stakes during the first two months (Photos by Ortega)

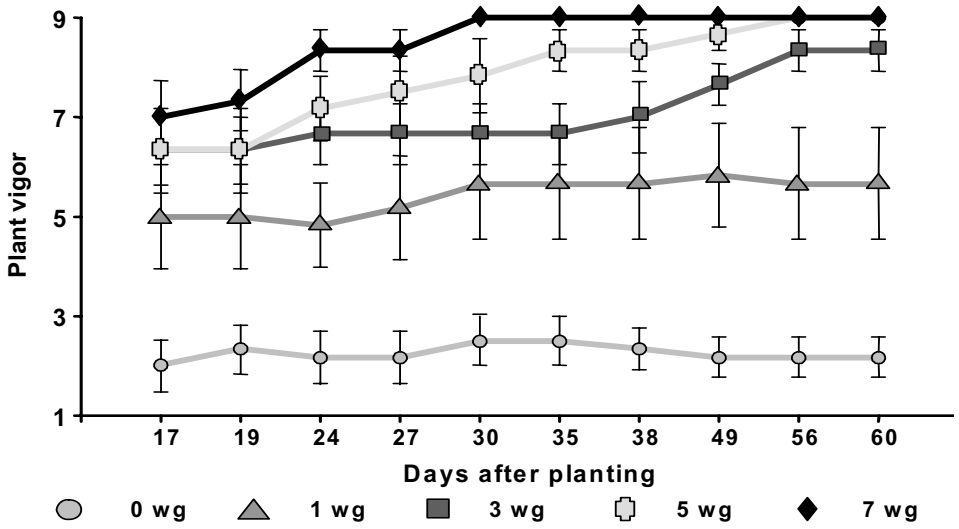

Figure 2.14.5. Vigor of cassava plants in relation to white grub (wg) density (Tukey pÒ0.05).

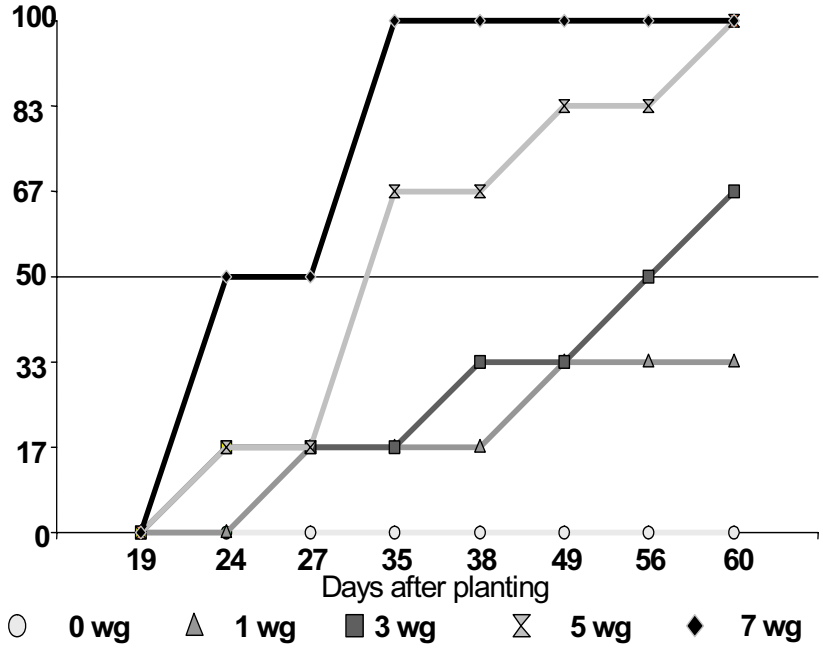

Figure 2.14.6. Probability of elimination of cassava plants in relation to duration of feeding (in days) and white grub density (Tukey pÒ0.05).

Activity 2.15. Pathogenicity of the bacteria *Paenibacillus popilliae* on larvae of *Phyllophaga menetriesi* (Coleoptera: Melolonthidae)

Contributors: Carolina Buitrago, James Montoya, Andreas Gaigl, Martha Londoño

Highlight:

- Highly virulent bacteria for the control of the white grub *Phyllophaga menetriesi* identified

Rationale

Bacteria traditionally play an important role as natural antagonists of insects, and particularly of white grubs. *Paenibacillus popilliae* is the pathogenic agent of milky disease and has been the subject of many studies. Its wide host range of about 70 white grub species (Tanada and Kaya, 1993, Insect Pathology, Academic Press Inc, California, USA) indicates this bacterium as an important component of biological control. In Colombia, the national agricultural research institution, Corporación Colombiana de Investigación Agropecuária (CORPOICA) in Rionegro, Antioquia, developed a methodology for artisanal on-farm rearing of *P. popilliae* in order to control *Phyllophaga obsoleta*, a key pest on potatoes in the agroecological cold zones of Antioquia between 1800 and 3000 m. However, no studies have been conducted on this bacterium as control agent of *P. menetriesi*, a key pest on cassava, maize, coffee, pasture, etc. in the 1000 to 1500 m.a.s.l. zone.

P. popilliae is also one of the most frequently identified microbial pathogens of *Phyllophaga menetriesi.* However, no experimental studies have been conducted of this bacterium as control agent of this grub. This present work aimed to test the pathogenicity of various strains of *P. popilliae* on *Phyllophaga menetriesi* and *P. bicolor*.

Materials and Methods

From the white grub-rearing colony at CIAT and CORPOICA in Rionegro, we selected six strains of *Paenibacillus popilliae* for the experiments. These bacteria were previously isolated from white grubs mainly collected in Pescador (Cauca, Colombia) (Table 2.15.1). Before initiating the experiments, we reactivated the isolates on agar L.

Table 2.15.1. Origin of the six *Paenibacillus popilliae* strains deployed in this experiment.

Strain	Site	Host
CIAT-LF24	Pescador (Cauca)	*Phyllophaga menetriesi*
CIAT-BP1	Pescador (Cauca)	*P. menetriesi*
CIAT-BP4	Pescador (Cauca)	*P. menetriesi*
CIAT-LG	Pescador (Cauca)	*P. menetriesi*
CIAT-381	Pereira (Risaralda)	*P. menetriesi*
Corpoica-B386	Rionegro (Antioquia)	*Ancognatha* sp.

Insects: For the assays for spore reproduction we used larvae of *Phyllophaga menetriesi* and *P. bicolor* in the third instar. We collected the insects on cassava and pasture fields in Pescador (Cauca, Colombia) and

quarantined for 8 weeks. For the pathogenicity bioassays we used larvae in the second instar taken from the white grub-rearing colony at CIAT.

Description of bioassays: We conducted four bioassays where we produced the inoculums, and one bioassay where we evaluated pathogenicity and virulence of the six strains in soil. We used two methodologies for the reproduction of inoculums: injection or forced feeding. After each bioassay, we verified the species of the bacteria according to Koch's postulates.

Reproduction of inoculums by injection and forced feeding: The experimental unit consisted of one larva of three white grub species—*Phyllophaga menetriesi*, *P. bicolor*, and *Anomala cincta*—confined in a 2-oz plastic cup, which was filled with sterilized soil and organic matter (1:1) at a humidity of 7% of field capacity. The grubs were fed with three maize grains. We used 50 larvae for each bacterial strain. The bacteria were introduced by injection into the third segment of the abdomen of the larva (10 µL suspension with 10^6 spores/ml). As control treatment we injected 10 µL distilled water into the insect. The treated larvae were checked every second day during 2 weeks. The insects were disinfected with alcohol and hypochlorite after each revision.

For the forced feeding of the grubs we developed an artificial diet that consisted of a mixture of healthy hemolymph, yeast extracts, beans, agar, NaCl, and inoculums. Prior to feeding, the larvae were starved for 48 hours. We revised the larvae in the third and sixth week after infestation.

After the extraction of hemolymph, we quantified spore concentration and validated the morphology of the bacteria (macroscopically and under microscope). This made it necessary to extract 0.1 ml of hemolymph from each larva that was diluted in 0.9 ml of distilled water. We then made serial dilutions until 10^6 spores/ml. These were cultivated in agar and counted using the colony forming unit (CFU).

Bioassay on pathogenicity of bacteria when applied on the soil surface: For the bioassay on pathogenicity and virulence we modified an experimental design described by Londoño *et al*. (2001, Technical Report, Rionegro, Antioquia, p 78) using 10 plastic cups of 16 oz each, with perforated lids. Each cup was filled with sterile soil and organic matter in a relation of 1:1, and some rice grains as food for the insects. We confined one larva (second instar) of *P. menetriesi* in each cup. The spores were applied in a solution of 0.5 ml and in two concentrations ($1x10^5$ and $1x10^8$) on the soil surface. Each experimental unit was stored in a dark room, at 23 °C and 70% RH. The suspensions were prepared on agar L. After 20 hours we harvested the colonies of each strain in Eppendorf tubes of 1.5 ml. These suspensions were diluted in 1 ml of sterile and distilled water, after which the suspension was ready for counting. Every dilution ($1x10^8$ spores/ml of distilled water, equivalent to 600 lambdas in the spectrophotometer) was poured into the Neubauer camera or hemacytometer to obtain the appropriate dilution of spores. These dilutions were measured with the spectrophotometer.

Results and Discussion

Reproduction of inoculums by injection and forced feeding: All dead insects showed a milky color under the cuticle. Only the larvae infested with BP1 were black; the mortality of the latter strain at 50% was not very high. We observed microscopically all the typical characteristics of bacterial spores. From these observations on BP1 we conclude that while spores penetrated into the gut they propelled the production of spores of other strains of *Paenibacillus popilliae* due to the well-known mechanism of antagonism between microorganisms. However, we also have to consider the possibility that this strain was contaminated with *P. lentimorbus*. Nevertheless, all dead larvae shared some characteristics, such as a soft and swollen body, smell of fermentation, the fact that after 24 hours all larvae turned black, and all responded to Koch's postulates.

When the bacteria were introduced by forced feeding, some diseased larvae showed typical symptoms of agony such as slow movements with white curd coloration. After the sixth week, the bodies turned soft, and then after a few days turned black. Microscopical examination of the hemolymph revealed that the endospores were shaped like a footprint. This is the typical sign for completing the fundamental cycle of infestation (Steinhaus, 1967, Principles of Insect pathology, Hafner Publishing Company, New York-London, 862 pp). In other words, all six strains were able to sporulate until the insect started the "bacteremia" completing the Koch postulates.

Most of the larvae in the second instar (soil experiment) were dead and decomposed after 8 weeks. The presence of bacteria was proven by soil analyses confirming macroscopically and microscopically the morphological characteristics.

The *P. popilliae* strain LF24 killed all larvae of all three white grub species. Strains 381 and LG performed with similar efficiency on *P. bicolor* and *P. menetriesi*, whereas on *A. cincta* these strains were less successful (Figure 2.15.1). Strain B386 was significantly less efficient on *P. menetriesi* than on the other two species. Surprisingly, LG caused a mortality of only 4% on *A. cincta*, whereas both *Phyllophaga* species were efficiently controlled (between 90% and 100%). The high mortality rate of *P. menetriesi* caused by B386 was not expected. This strain was isolated from a white grub of the genus *Ancognatha*. These white grubs are endemic in agroecological zones above 2400 m, a much colder region where *P. menetriesi* is endemic. However, there are several cases where microbial organisms obtained efficient control of white grubs although they do not share the same agroecological zones. Shannon and Carballo (1996, CATIE Informe Tecnico No. 227, Costa Rica) found that the entomopathogenic fungus *Metarhizium anisopliae* was the most efficient antagonist of *Phyllophaga vicina* although both are endemic in different zones. These authors concluded that exotic entomopathogens could develop more efficiency as control agent than endemic ones that are intimately related to both host plant and insect.

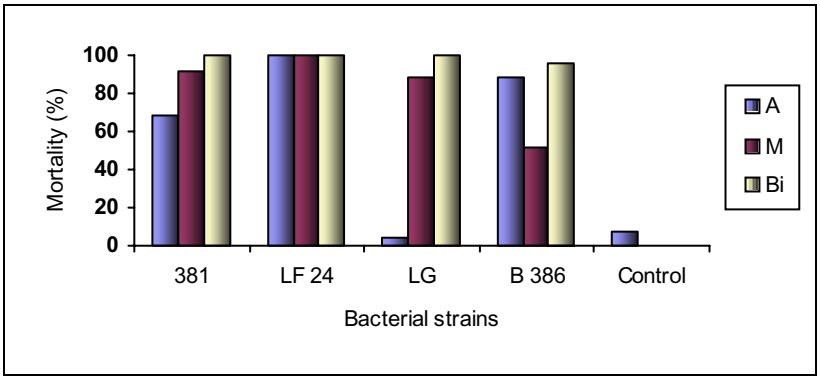

Figure 2.15.1. Mortality of four bacterial strains on three white grub species (second instar): A = *Anomala cincta*; M = *Phyllophaga menetriesi*; Bi = *Phyllophaga bicolor*.

All six bacterial strains caused a mortality of larvae of *P. bicolor* between 90% and 100% (Figure 2.15.2) suggesting that all isolates are a promising tool for the control of this white grub. Interestingly, only the strain BP1 was isolated from *P. bicolor*; nevertheless, all other strains were highly pathogenic on this white grub. These results also corroborate Shannon and Carballo (1996, CATIE Informe Tecnico No. 227, Costa Rica) who observed an increased pathogenicity of exotic entomopathogenic fungi on white grubs.

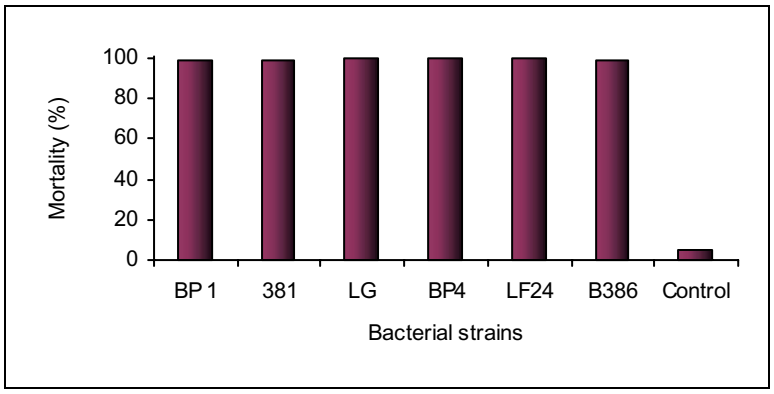

Figure 2.15.2. Mortality of larvae of *Phyllophaga bicolor* after injection of six strains of *Paenibacillus popilliae*.

Pathogenicity of the bacteria on *P. menetriesi* was similar to the experiment with *P. bicolor* (Figure 2.15.3). All strains must be considered as promising control agents of this pest. As expected, we obtained the highest mortality of grubs when the bacteria were injected. After the fourth day we found those larvae infested with LF24, BP1, B386, and 381were considerably diseased. Nevertheless, these larvae continued feeding. Beard (1945, Conn. Agr. Exp. Sta. Bull. 491: 505-83) made a similar observation, showing that white grubs do not die in a defined moment after an attack by entomopathogens, and that the time taken depends on the vigor of each individual. It is even possible that white grubs diseased by *P. popilliae* or *P. lentimorbus* may molt into pupa or adults, although with deformations.

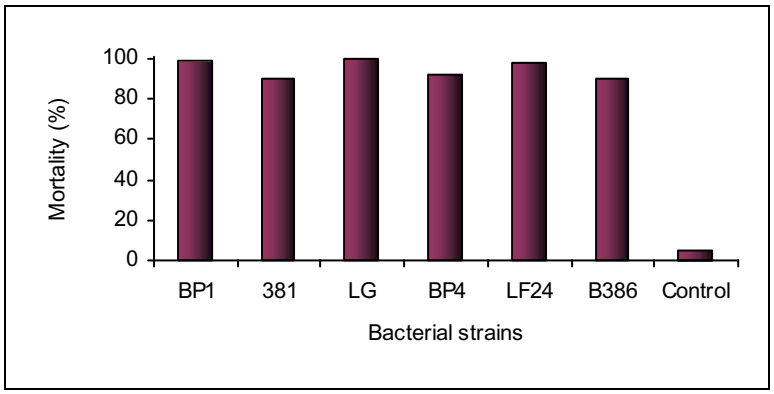

Figure 2.15.3. Mortality of larvae of *Phyllophaga menetriesi* after injection of six strains of *Paenibacillus popilliae*.

When forced feeding was used on the larvae, 50% of them died in the third week, except for BP1 and B386. The range of mortality showed a wide variance (42% to 87%). The most efficient strains were LF24 (87%) and BP4 (79%) (Figure 2.15.4). Comparing the results between forced feeding and injection, we conclude that the behavior of the bacteria varies more when the penetration succeeded in a natural way (by feeding).

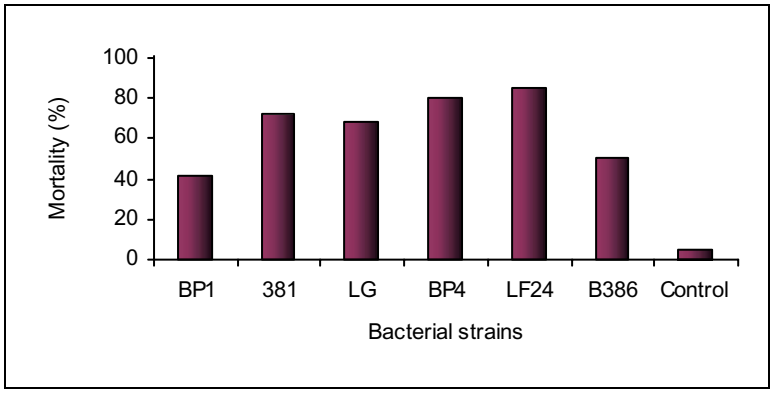

Figure 2.15.4. Mortality of *Phyllophaga menetriesi* when *Paenibacillus popilliae* penetrated the insect by forced feeding.

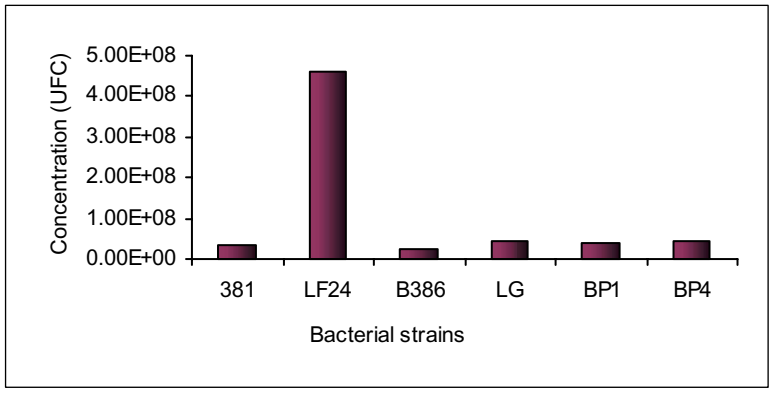

Figure 2.15.5. Cell concentration (UFC) per ml of six strains of *Paenibacillus popilliae* reproduced in *Phyllophaga menetriesi.* Bacteria applied by injection.

In these experiments, we included the commercial strain "Doom Japidemic", which is used in the US against the white grub *Popillia japonica*. This product caused only a poor mortality of *Phyllophaga menetriesi*, corroborating the findings of Diaz (1992, Msc thesis, CATIE, Costa Rica, 67 p) and Londoño (2002, In: Control biológico: Componente fundamental de manejo integrado de plagas en una agricultura sostenible, Lopez-Avila (ed), CORPOICA, Bogota, p 40-48). Following our observations, we share the opinion of Klein and Jackson (1992, In: Use of pathogens in scarab pest management, Jackson and Glare (eds), Intercept, Andover, UK, p 43-62) that tests on cross infectivity between species normally yield negative results. Hence, we suggest not including this strain in further studies on biological control of *P. menetriesi*.

Reproduction of inoculums by injection and forced feeding: As expected, mortality and reproduction rate of inoculums is directly proportional. Both *P. bicolor* and *P. menetriesi* presented high levels of spores in their hemolymph when mortality was high. In general, we obtained greater mortality when spores were injected than by forced feeding. Our results on feeding are similar to studies by Hidalgo *et al*. (1998, Avances en el estudio de la diversidad, importancia y manejo de los coleópteros edafícolas americanos, Universidad Autonoma de Puebla y la Sociedad Mexicana de Entomologia, Mexico, pp165-172) who obtained a 29% mortality of *P. menetriesi* when they applied $1x10^7$ spores per larva.

LF24 performed outstanding cell reproduction when bacteria were applied by injection (Figure 2.15.5). When we introduced the bacteria into the grub by forced feeding, LF24 gave the best reproduction; however, difference to other strains was not as pronounced as in the previous experiment (Figure 2.15.6). Obviously, the bacteria reproduced much better when they were injected. The natural form of penetration can explain this—the bacteria entered with food through the mouth, and the insect ingested them naturally. Subsequently, they penetrated the intestinal wall, germinated in the lumen, and entered the hemolymph where they finally caused the bacteremia. We conclude that the penetration into the intestinal wall is a decisive step in the infection process and recommend applying this methodology for future research on milky disease.

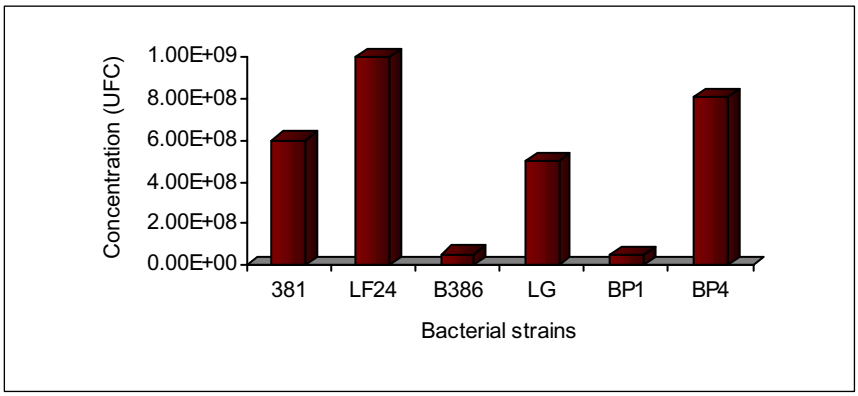

Figure 2.15.6. Cell concentration (UFC) per ml of six strains of *Paenibacillus popilliae* reproduced in *Phyllophaga menetriesi*. Bacteria applied by forced feeding.

Bioassay on pathogenicity of bacteria when applied on the soil surface: Since we did not observe any significant differences between the concentrations of each strain we present all data of this experiment in one chart. LF24 caused a white grub mortality of 83% and was by far the most efficient bacterial strain (Figure 2.15.7); the second strain, BP4, only controlled 51%.

These data corroborate the results of the experiment of reproduction of inoculums by forced feeding and stress the outstanding efficiency of LF24 followed by BP4. Moreover, our experiments suggest that strain LF24 in particular is a highly promising candidate as biopesticide of white grubs and that it is effective against second and third instar of *P. menetriesi*. Other microbial entomopathogens such as nematodes (unpublished data) or fungi did not obtain satisfying results when they were applied on the third instar of this species, even when applied at extremely high dose directly on the insect (10,000 nematodes/ml) As in the case of nematodes, the combinations with other microbial pathogens or insecticides (e.g., imidacloprid or fipronil at sublethal dose) may even improve efficiency of this bacterium.

We hypothesize that two circumstances favored this bacterial strain: First, the inoculums were extracted from the same host (*P. menetriesi*); and second, the spores were recently extracted from white grubs.

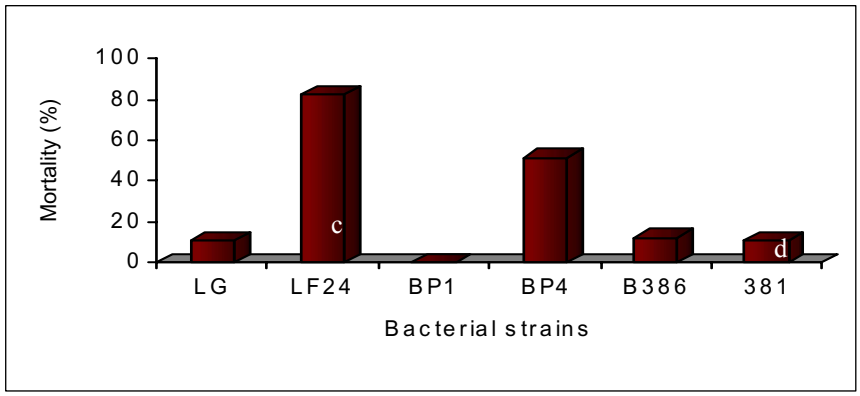

Figure 2.15.7. Mortality of second larval instar of *Phyllophaga menetriesi* when bacteria were applied on the soil surface of the experimental unit.

In the near future, LF24 should be tested in the greenhouse and in field experiments. Moreover, other bacterial strains should be tested on each developmental stage of *P. menetriesi*. The combination with other pathogens such as nematodes or fungi may enhance efficiency of the bacteria.

Activity 2.16. Estimating grade of damage caused by the soil pests *Phyllophaga* spp. (Coleoptera: Melolonthidae) in maize, beans and cassava

Contributors: Alberto Ortega-Ojeda, Elsa Liliana Melo-Molina, Andreas Gaigl

Highlights:

- Economic threshold of white grub species *Phyllophaga menetriesi* on three crops defined
- Tool developed to detect white grub attack by plant phenology.

Rationale

Maize (*Zea mays* L.), beans (*Phaseolus vulgaris* L.), and cassava (*Manihot esculenta* Crantz) are three of the principal tropical crops grown at north of the Department of Cauca in Colombia after sugar cane (*Sacharum officinalis*), according to surveys carried out with farmers of the area (unpublished data). These crops are severely affected by white grubs or *chisas* when these attack during the crops' first month of establishment. These rhizophagous (root-eating) pests from the Melolonthidae family (Coleoptera) have evolved a wide diversity of species associated with different thermal floors and agroecosystems in Colombia. The greatest diversity of white grubs in southwestern Colombia is in Caldono, Department of Cauca, where the white grubs of greatest economic interest include several species of *Phyllophaga*, *Anomala inconstans*, *Cyclocephala lunulata*, *C. fulgurata*, *Plectris fassli*, and *P. pavida*. As many as 5 larvae per square meter were observed in cassava and coffee crops. Among them, *P. menetriesi* is the most important pest because its third-instar larvae are long, reaching 4 cm. They are also voracious, aggressively consuming roots of a large variety of cultivated plants and causing significant crop losses.

Most damage to annual crops such as maize and common bean occurs when the insect attacks the plant, close to where the stem meets the root. The plants present little development with symptoms of wilting

and chlorosis as consequence of root losses, in accordance to the observations by the authors in field and greenhouse experiments between 2003 and 2005 (King, 1984, Tropical Pest Management 30: 36-50).

White grub damage on cassava is verified by consumption of the stake, of its roots, cortex and young outbreaks. Some reports even mention perforated swollen tubers of mature plants in Panama and in the Departments of Quindío and Casanare in Colombia. The harm of the rhizophagous escapes notice, as there has verified it the author in crops of cassava, due to its ground development, which means that the farmer replaces fruitlessly its plants of cassava affected with new stakes, achieving only to continue the feeding of the white grub and increasing an additional cost of seed to the original losses.

Most damage done to annual crops such as maize and beans can be seen as well-defined patches of sick plants, or plants with sick parts, on the farm. The insect attacks the plants close to where the stems and roots join. The plants develop little, show wilting, and change color as they lose roots and so suffer nutrient and water deficiencies. These field observations in Cauca corroborated findings by King (1984, Tropical Pest Management 30: 36-50), and were supported by greenhouse experiments carried out over 2003–2005 in Valle del Cauca.

In cassava, damage by white grubs is confirmed by consumption of stakes, their rootlets and roots, bark, and young shoots. Some reports in Panama even mention attacks on mature plants, where one to three large perforations were found in thickened roots. Perforating galleries were also observed in cassava roots in the Departments of Quindío and Casanare of Colombia.

Damage by rhizophagous escapes notice because of their underground development. The farmer fruitlessly replaces affected cassava plants with new stakes, only to continue feeding the white grubs and add further seed costs to the original losses. In seeking fast solutions, some farmers use synthetic pesticides but with unsatisfactory results and at the cost of their own and the environment's health, as well as the product's own high costs. Such products also tend to generate resistance in the pests, forcing farmers to increase dosage with the consequent increase in the problems just mentioned.

A few authors suggest, based only on their observations, that the possible economic threshold (level of pest infestation at which farmers should take action to prevent significant crop losses) was 3 larvae per plant. However, the monitoring tool used to manage the pest lacks an indicator for deciding when to take control measures over the insect. We therefore proposed to establish, for the three crops mentioned above, a relationship between the degree of visible damage to the plant in the first month of establishment and the presence of *Phyllophaga* larvae.

In contrast to previous studies where the experimental unit for infestation was artificial, we conducted this experiment in the field under natural infestation. This innovative challenge was intended to contribute knowledge on the dynamics of the pest with its host crops. The study aimed to phenotypically establish degrees of damage in the young plant that would tell the farmer when to apply control measures for *Phyllophaga*; quantify potential yield losses in terms of the initial damage done by the rhizophage; and, in the case of cassava, evaluate the damage done by a second generation of larvae that develop during the crop's stage of maturity and thus verify reports of damage in this stage. For the bean and maize crops, whose maturity stage did not coincide with a new generation of *Phyllophaga*, we evaluated the effect of other white-grub genera on the final yield.

Materials and Methods

The experiment was carried out at Bellavista Farm, located in the Village District of Pescador, Municipality of Caldono, Cauca, at 1580 m above sea level (2°49'15.1" N and 76°33'45.6" W). We

followed recommended cultural practices in planting and raising maize, bean, and cassava crops. Maize was planted on 1200 m^2, using variety ICA V-305 at a density of 37,500 plants/ha. Bean variety ICA-Toné was also planted on 1200 m^2 but at a density of 90,000 plants/ha. Cassava variety SM 707-17 was planted on 1300 m^2 at a density of 10,000 plants/ha. The plots lay in an area usually infested by white grubs, as confirmed by a preliminary random sampling of soil. The insects were disturbed as minimally as possible and, after sampling, were left where they were found.

The study aimed to classify the health of young plants, between 0 and 30 days old, according a phenotypical scale based on agronomic parameters to determine damage done by white grubs. Visual criteria were classified into grades 1, 3, 5, 7, and 9, where 1 corresponded to a healthy plant (ideal) and 9 to an agronomically irrecoverable plant (Table 2.16.1).

Table 2.16.1. Phenotypical scale based on the agronomic value for determination of treatments in young plants of maize, to the 30 days from the seeding**.**

Level	Plant	Correspondence
1	Excellent (Control)	Ideal plants, with excellent architecture (stems, petioles and erect leaves); leaves dark green color; good vegetative development (Ó20 cm).
3	Good	Plants with good architecture but smaller height (Ø15 and <20 cm) slightly thinner; smaller number of leaves that the previous.
5	Intermediate	Plants with poor architecture, mild chlorosis; less leaves that the previous; stalk lengthØ10 and <15 cm, thin.
7	Poor	Chlorotic and rachitic plants (poor architecture, weak stalksØ5 and <10 cm; few and small leaves.
9	Very Poor	Irrecoverable plants; without or very limited and small leaves; very chlorotic and/or wilted stalks, with length <5 cm

One plot with 10 planting sites was chosen for each of the 5 grades (i.e., one experimental unit of 1 m^2 x 10 replications × 5 = 50 sites). The total experimental area was therefore 500 m^2. The size of the unit (1 m^2) was chosen according to the recommendation for capturing white grubs. Each site was planted with 3 plants of maize, 9 of beans, and 1 of cassava. A randomized complete block design was used with a unifactorial arrangement. Evaluations were made at 30, 90, and, for cassava, 400 days after planting (DAP). After an analysis of variance (Anova) the Tukey's Multiple Comparisons Test (P Ò0.05) was carried out for the significant variables. Before statistical analysis data were transformed by $\sqrt{x\ 2\ 1}$.

The sale prices used for the economic analyses of the maize and bean crops were provided by the Prices Information Service of the Agricultural and Livestock Sector (SIPSA 2005). The following prices were used (25 June to 1 July 2005): for threshed dry grain of maize, Col$733/kg; and dry grain of beans, $3088/kg. For cassava, two calculations based on sales were made: one for the starch industry, where a sales value of Col$340/kg (wt/kg) was calculated because variety SM 707-17 was produced in a "cold", not "hot", climate, which otherwise would have cost $250/kg. The other calculation was based on the fresh-root market, for which the price $400/kg was calculated.

Results and Discussion

Maize: The height of the plants was strictly related to the density of the white grubs (pÒ0.05) (Figure 2.16.1a). In the absence of the pest the plants reached a height of 15 cm 30 days after planting (DAP) and

275 cm 90 DAP. In contrast the plants that were associated with many grubs reached an altitude of only 125 cm 90 DAP. The typical average height of this variety is 234cm (Navas *et al*., 1993, Fitotecnia Colombiana 4:55-65) indicating that the crop was accurately managed during this experiment. When the damage is of intermediate level or more plant height (181.9 cm) is significantly lower.

The number of maize plants did not show any differences among the treatments (white grub densities) at 30 DAP (pÒ0.05), although differences in agronomic value were present. This is because the white grubs first fed on roots and then on the plant's hypocotyl, which meant that no losses of plants were yet seen (Figures 2.16.1b). At 90 DAP the plants showed advanced damage scoring 5 and 7. Control plants and those with minimal damage (level 3) had an average of 3.3 plants, in contrast to plants with damage at level 5 or more (plants of intermediate appearance up to irrecoverable), which had an average of 1.3 plants per site after initially damaging roots and rootlets, *P. menetriesi* had changed plants, thus significantly reducing (*P* Ò0.009) the severity of damage (Figure 2.16.1b). This contrasts with what was observed at 30 DAP.

Insertion heights for ears reached 145.0 cm in control plants, whereas, in plants with the worst degree of damage, the height was only 32.3 cm. The other treatments had an average of 117.2 cm. The varietal mean was 130 cm (Navas *et al*., 1993, Fitotecnia Colombiana 4:55-65). Our findings therefore suggested that the genotype was influenced by all degrees of damage, except for the control. Moreover, this variable correlated strongly with plant height (Figure 2.16.1a), as was expected from what is known of this crop's genetic patterns.

The major diameters of the stalk (2.63 cm max. and 2.17 cm. min.) corresponded to the scales of damage 1 (control), 3, and 5 of harm (mild and intermediate) (Figure 2.16.1c). There was a clear tendency that the diameter of stalk diminished while damage increased.

The thickest stem diameters (2.17 to 2.63 cm) correlated with scores 1 (control), 3 (minor damage), and 5 (moderate damage) (Figure 2.16.1c). The trend clearly showed that the stems became thinner as damage increased. This variable is important in that it can influence lodging to a lesser or greater extent and, hence, indirectly affect yield.

The heaviest ear corn weight was presented by the control at 358.7 g, in contrast to the average of 78.7g of treatments 2 to 5 (degree of damage 3 to 9) (Figure 2.16.2a). That is, an obvious inversely proportional trend is shown between yield in ear weight per plant and severity of damage.

Results for dry grain yield in maize were similar to those for the ears, with the control distancing itself at a yield of 305.4 g (Figure 2.16.2a) from all the other treatments. The results for this and the previous variables are remarkable, as they are direct indicators of yield, which is affected, even though no drastic impact was observed for such important variables as the number of plants per treatment in the crop's early stages.

Likewise, the 100-seed weight in maize showed a clear trend to drop as plant damage increased (Tukey's; *P* Ò0.05). The best treatments were the control and the least degree of damage, with an average of 38.8 g. These contrasted with the unstable intermediate treatment (degree of damage 5), which had 30.6 g, and the worst degrees of damage, 7 with 23.0 g and 9 with 2.63 g (Figure 2.16.2b). On relating these results with those of the previous two variables, we can infer that, although the first three levels of damage were statistically similar for 100-seed weight, the quantity of grain produced by the control (largest number of ears per plant) sets it at a distance from the other two. Hence, the minimal presence of the rhizophage must clearly be avoided to prevent yield loss. Moreover, to maintain only a 5% loss, measures need to be taken when 3 in 50 sites per hectare (chosen at random) are found to have at least one white grub each (assuming a density of 30,000 plants/ha).

Mature maize plants did not suffer damage. Larvae of *P. menetriesi* had already become pupae by harvest and were located at a depth of 30 cm. Any larvae found corresponded mainly to *Astaena* sp., *Cyclocephala* sp., *Anomala* sp., *Plectris fassli*, and *P. pavida*, as identified according the form of the palidia in their rasters. These larvae were not as aggressive as *P. menetriesi*, which meant that any damage they caused did not reflect in the crop. In addition, it can be stressed that the presence of white grubs was correlated with increasing agronomic values (Figure 2.16.2c) (Tukey pÒ0.05). We hypothesize that female beetles prefer to oviposit near healthy plants to ensure their progeny survival. This population, however, did not cause any significant damage since the crop was going to complete its cycle. This contrasts with Posada's findings (1993, Agricultura Tropical 30: 71-79), where, in their most advanced stages of development, crops would show yellowing and even lodging.

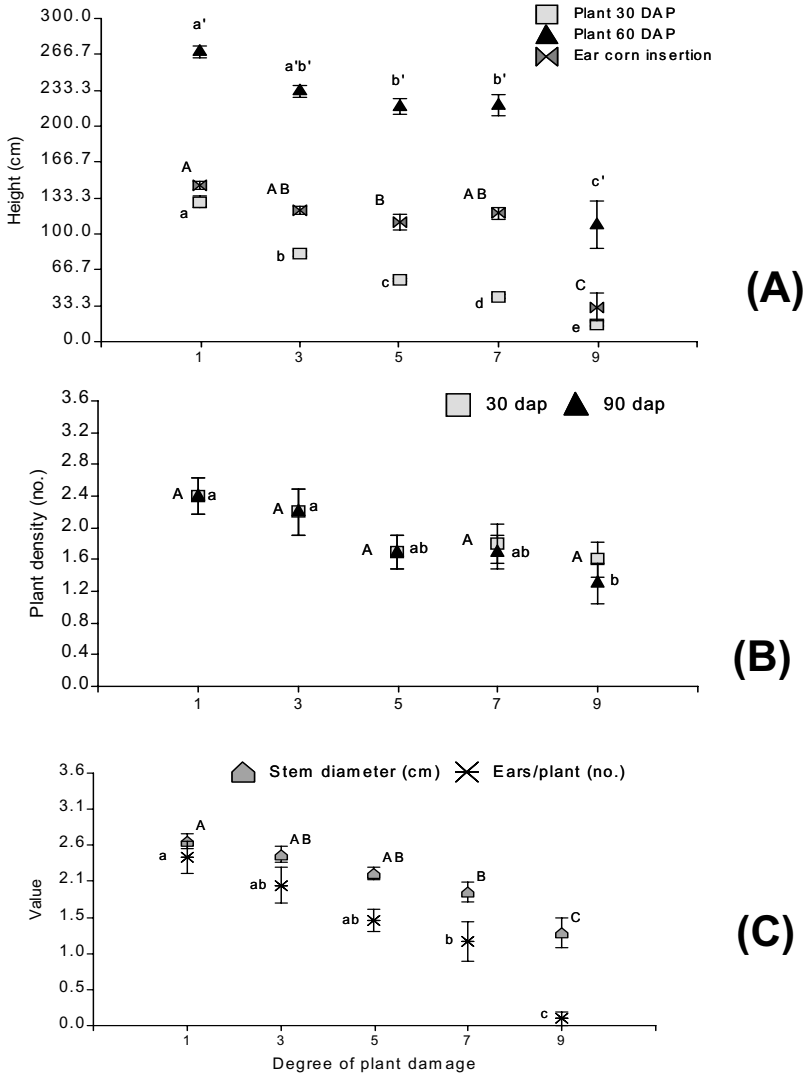

Figure 2.16.1. a) Altitude of plants to the 30 DAP (days after planting) and 90 DAP (physiological maturity) and Altitude of insertion of ear of corn (90 dap); **b**) Number of plants by site to the 30 DAP and to physiological maturity (90 dap); **c**) Diameter of stalk (cm) and number of ear of corns by plant to physiological maturity (u) (Tukey pÒ0,05)

The economic analysis of dry grain yield of maize can be appreciated in Table 2.16.2. Even with the least observed damage, losses were catastrophic (50%). This finding suggests that when farmers detect any damage, they need to eliminate the white grubs, and re-plant the crop.

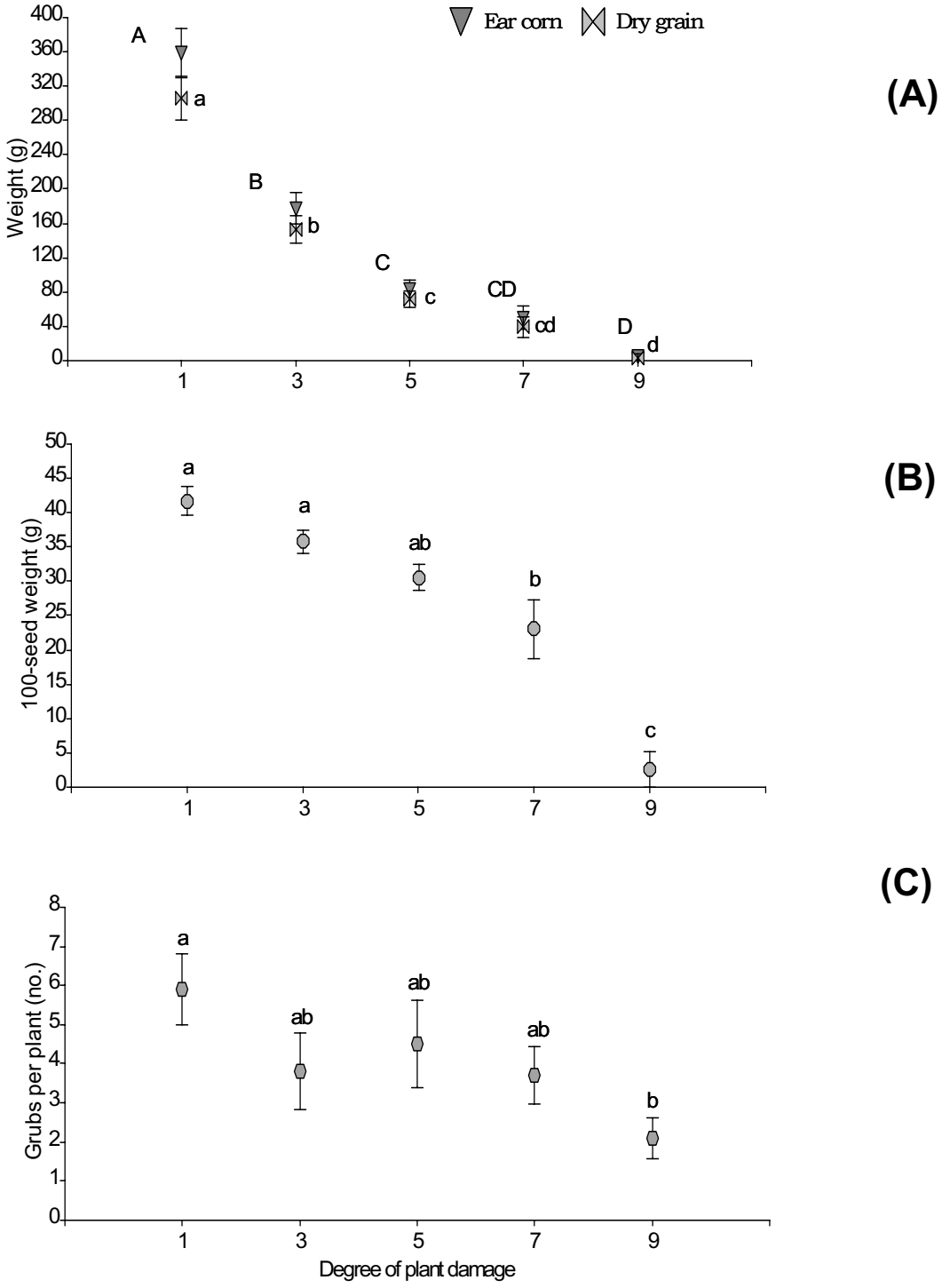

Figure 2.16.2. Evaluated variables: **a.** Yield, in grams, of ear of corns by plant and of dry grain, to the harvest (150 days after planting); **b.** Weight of 100 seeds in dry grain (g); **c.** Number of white grubs of second generation by plant and by treatment (Tukey pÒ0,05).

Beans: None of the affected plants reached the height of the controls (pÒ0.05) indicating that even the least damage (level 3) during the initial phase affects significantly the development of the crop, although the number of plants per experimental unit did not decrease (Figure 2.16.3).

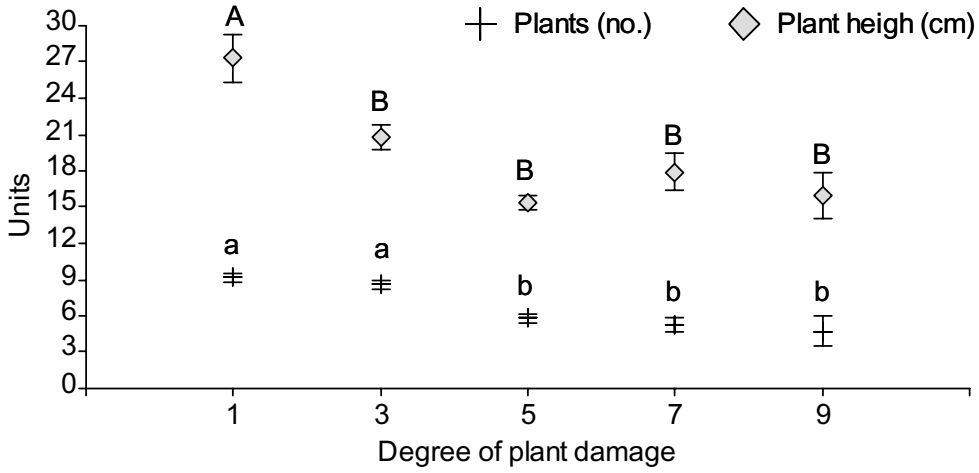

Figure 2.16.3. Number and height of plants of shrub-like beans per square meter (treatment), to the harvest in dry grain (Tukey pÒ0,05).

None of the control plants and of those of with minimal damage suffered any plant losses, unlike the others with damage classified as 5 to 9 (Figure 2.16.3) that were associated with an increased number of white grubs. However, the weakest damage was product of consumption of the roots by white grubs affecting yield, even though the elimination of plants was not notable (pÒ0.05).

The differences in the losses of the dry grain (g) corresponded to the different degrees of damage (Figure 2.16.4). Losses reached 39.8% even when visible damage was minimal, worsening with each higher degree of damage evaluated (leves 5, 7 and 9). From these results and reasoning businesslike, we recommend permitting only a white grub by plant in maximum 5% of the population of the crop of beans.

At the end of May and beginning of June we found a population of white grubs that was composed by the genera and species *Plectris* spp., *Cyclocephala* spp., *Anomala* spp., and *Phyllophaga bicolor*. These species are economically less important than *P. menetriesi*. In spite of the white grub diversity there were not differences between levels of estimated damage, which made it possible to conclude that other white grubs than *Phyllophaga menetriesi* did not affect the yield of beans. Hence, we recommend to plant beans when *P. menetriesi* completes the larval stage in order to elude its attacks.

In bean crops, the economic losses caused by even the least degree of damage can reach 60% (Table 2.16.2). This means that, to reduce losses, damage must be prevented and/or the crop quickly re-planted after measures have been taken against the rhizophagous.

Table 2.16.2. Ratio of gains and losses per kilogram of grains according to different degrees of damage by white grubs to plants growing in a commercial plot of beans.

Damage[a]	Yield (kg)	Income (Col$/kg)	Losses	
			(Col$/kg)	(%)
1	0.131	440.19	0.00	0.00
3	0.052	175.02	265.17	60.24
5	0.037	123.65	316.55	71.91
7	0.034	113.60	326.59	74.19
9	0.015	50.13	390.06	88.61

a. On a scale of 1 to 9, where 1 = no damage (control) and 9 = severe damage (agronomically unrecoverable plant).

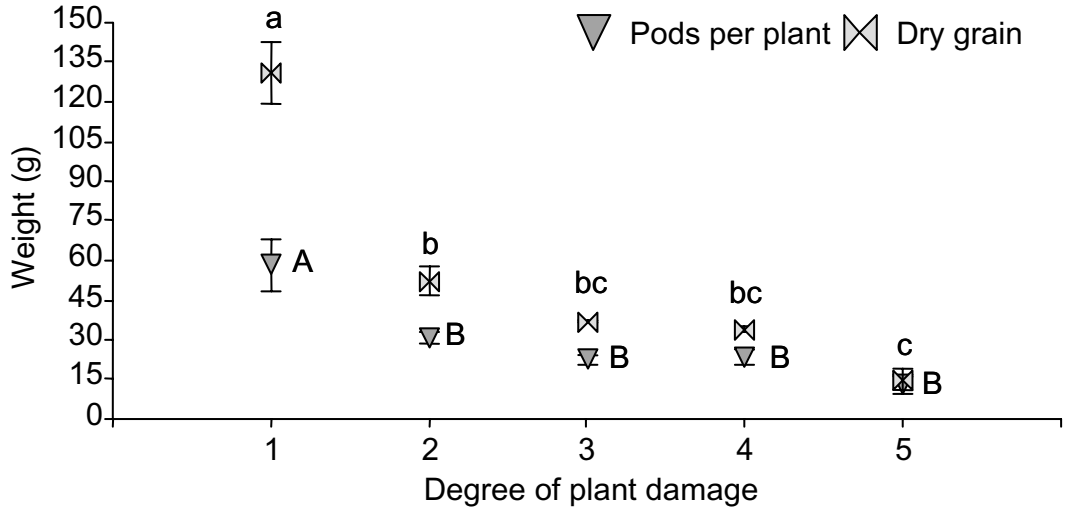

Figure 2.16.4. Number of pods per plant and yield of dry grain (g) in bush beans per square meter (treatment). Bars indicate standard errors (Tukey's; *P* Ò 0.05). The same letters per variable indicate that the values are not statistically different to each other

Cassava: In this long-cycle crop, we observed that plants with the least degree of damage (grade 3) improve over time, whereas those suffering moderate (grade 5) to severe damage (grade 9) tend to worsen (Figure 2.16.5a). In other words, the cassava plant can recover after an initial, temporary, and minimal damage to the propagule (stake and root system). The final yield will have been directly compromised in proportion to the degree of initial damage.

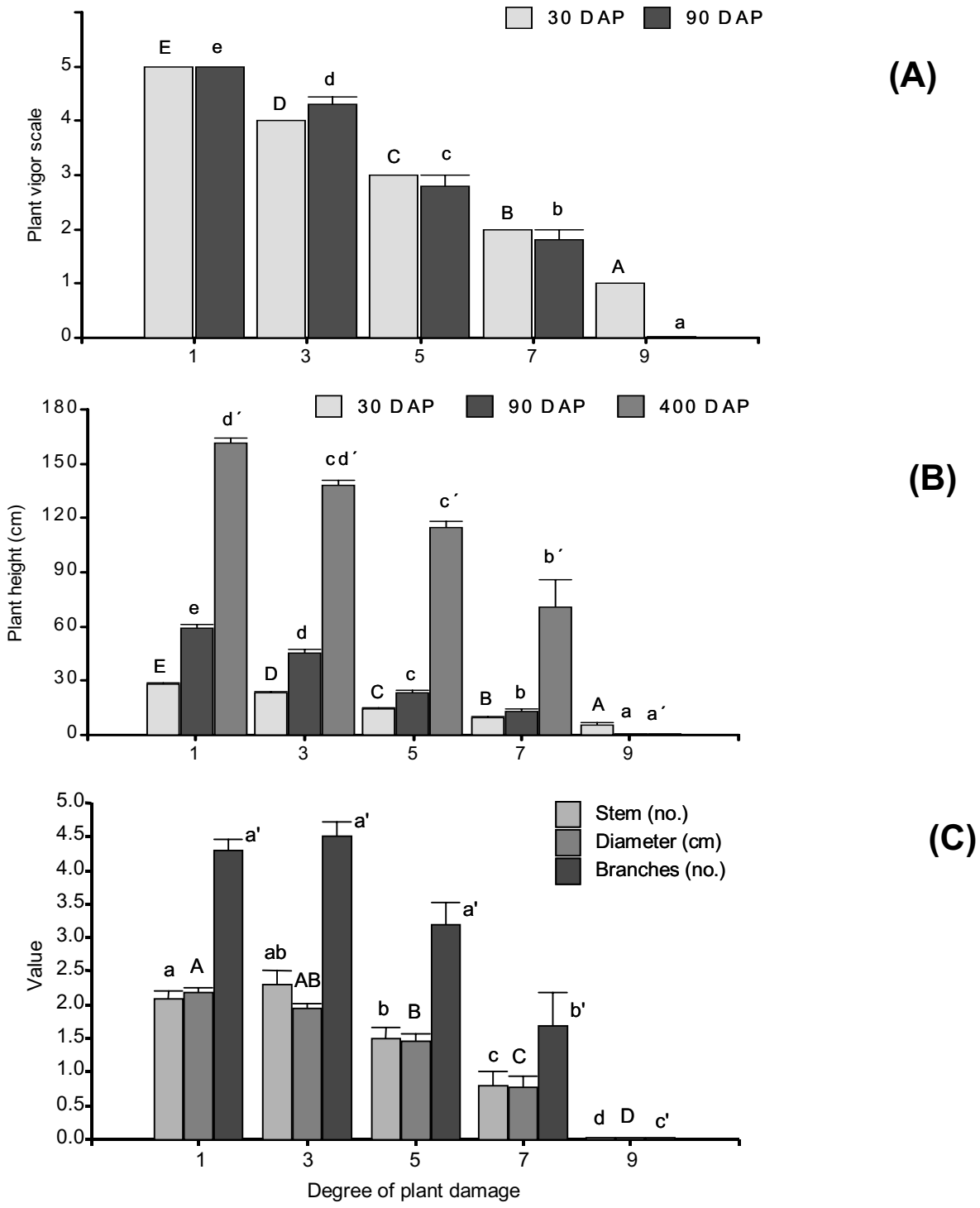

Figure 2.16.5. Evaluated variables for cassava: (A) plant vigor at 30 and 90 days after planting (DAP), on a scale where 0 indicates dead plant and 5, very vigorous plant; (B) plant height at 30, 90, and 400 DAP; and (C) average number of stems and branches per plant at 400 DAP. Bars for standard errors are shown (Tukey's; *P* Ò 0.05). The same letters per variable indicate that the values are not statistically different to each other.

At 90 DAP, no damaged cassava plants (whether scoring 1 or 9) reached the height of the control plants, thus showing that even the least degree of damage during crop establishment significantly affects vegetative development. However, at 400 DAP, plants scoring 3 for damage attained a height of 1.62 cm, like the control plants (*P* Ò 0.0001), with a maximum height of 1.62 cm (control). This finding corroborates the theory that if the plant survives and can recover well, once the larva stops feeding and passes to the prepupal stage (Figure 2.16.5b).

Stem number and diameter are similar until the degree of damage is 3, that is, these genotypic traits are not observed as affected if damage is minimal. For damage grades 1 and 3, the highest averages for diameter and number of stems (Figure 2.16.5c) are 2.1 cm and 2.2 stems, respectively. The other damage grades (5 y 7) produced averages of 1.2 stems and 1.1 cm of diameter. The plants classified as level 9 died (*P* Ò0.0001).

Plants with degrees of damage 1 and 3 were characterized by an average of 4.4 branches (Figure 2.16.5c). However, from 5, the number of branches declined significantly, which would occur if more than one white grub feeds on the propagule.

For the total number of roots, both commercial and noncommercial, we observed significant differences corresponding to the degree of damage (Figure 2.16.6a). The control produced 19.5 roots per plant, whereas plants with minimal damage produced only 13.4 roots, the number dropping to zero as level 9 was reached. If the cassava was destined for the starch market, the economic losses would be significant (*P* Ò0.0001).

In contrast to the previous variable, the number of only commercial roots did not differ significantly between the control and least damage (grade 3), which suggests that if the attack is light, then the plant recovers and its yield is satisfactory for the fresh-root market (Figure 2.16.6b). The maximum number of commercial roots per plant was 7.2 for control plants, followed by 5.3 for plants scoring 3 for damage (Figure 2.16.6a). These values surpass the variety's historical average of 4.0 commercial roots per plant. This indicates that the soil's nutritive conditions and the climate were favorable and did not act as covariables in the results. Where the degree of damage was 9, no commercial roots were produced.

The maximum yield in terms of weight of *all* roots was obtained by the control plants with 4.3 kg/plant, followed by plants scoring 3 for damage with 3.0 kg/plant (*P* Ò 0.0001). These values surpass the variety's historical average of 2.4 kg/plant. Meanwhile, plants scoring 5 and 7 damage produced an average of 0.6 kg of roots. Level 9 did not have plants (Figures 2.16.6a and 2.16.6b).

Unlike the previous variable, yield in terms of weight of *commercial* roots was similar for healthy plants and those suffering minimal damage, averaging 2.6 kg /plant (Figure 2.16.6b). Again, this finding suggests that, if the initial damage is limited, the plant can recover and produce equally to a healthy plant. According to our observation this might happen when:

- the larvae have sufficient space and can continue feeding on other plants
- the stake is planted when the larvae are finalizing instar III
- the plant has surpassed the first month of its development before larvae of *P. menetriesi* in the third instar.

We hypothesize that, where damage is minimal, a given larva had had sufficient space to travel through the soil to feed on more than one stake during its development, or the stake was planted at the end of third instar when the rhizophage could do little damage to the propagule, or the plant was more than 1 month old before the third-instar larva reached it.

As we mentioned with regard to maize and beans, we recommend that losses should not exceed 5% before taking control measures against white grubs, that is, when 3 out of 50 randomly selected planting sites are found with larvae. Also, because the samples of *Phyllophaga* larvae on farms did not show generalized infestations, measures should be applied after sequential samplings to discover where the infested areas are (Velásquez, 1994, CATIE Informe Técnico No. 277, Costa Rica).

*Economic analysis of damage of cassava roots***:** The economic losses incurred for plants with even the least damage reached almost 26% for the fresh-root market, whereas for the starch market, losses reached almost 30% (Table 2.16.3). These losses are sufficiently significant to oblige, as for maize and beans, roguing the crop, eliminating the white grubs, and re-planting the crop immediately. Losses incurred by moderate degrees of damage exceed even these percentages.

The economic losses incurred for plants with even the least damage reached almost 26% for the fresh-root market, whereas for the starch market, losses reached almost 30% (Table 2.16.4). These losses are sufficiently significant to oblige, as for maize and beans, roguing the crop, eliminating the white grubs, and re-planting the crop immediately. Losses incurred by moderate degrees of damage exceed even these percentages.

Table 2.16.3. Relation of gains and losses per kg of root according to each treatment.

Treat.*	Yield (kg)		Entries ($)		Losses			
	Total	Commercial	Sold for starch	Sold in fresh	Sold for starch ($)	Sold in fresh ($)	Sold for starch (%)	Sold in fresh (%)
1	4,28	2,98	1455,20	1192,00	0,00	0,00	0,00	0,00
2	3,02	2,21	1026,80	884,00	428,40	308,00	29,44	25,84
3	1,06	0,73	360,40	292,00	1094,80	900,00	75,23	75,50
4	0,13	0,00	44,20	0,00	1411,00	1192,00	96,96	100,00
5	0,00	0,00	0,00	0,00	1455,20	1192,00	100,00	100,00

* Treat. = Treatment

Table 2.16.4. Relation of gains and losses per kg of root of cassava according to different levels of harm in plants of a commercial lot.

Treat. *	Yield (kg)		Entries ($)		Losses			
	Total	Commercial	Sold for starch	Sold in fresh	Sold for starch ($)	Sold in fresh ($)	Sold for starch (%)	Sold in fresh (%)
1	4,28	2,98	1455,20	1192,00	0,00	0,00	0,00	0,00
2	3,02	2,21	1026,80	884,00	428,40	308,00	29,44	25,84
3	1,06	0,73	360,40	292,00	1094,80	900,00	75,23	75,50
4	0,13	0,00	44,20	0,00	1411,00	1192,00	96,96	100,00
5	0,00	0,00	0,00	0,00	1455,20	1192,00	100,00	100,00

* Treat. = Treatment

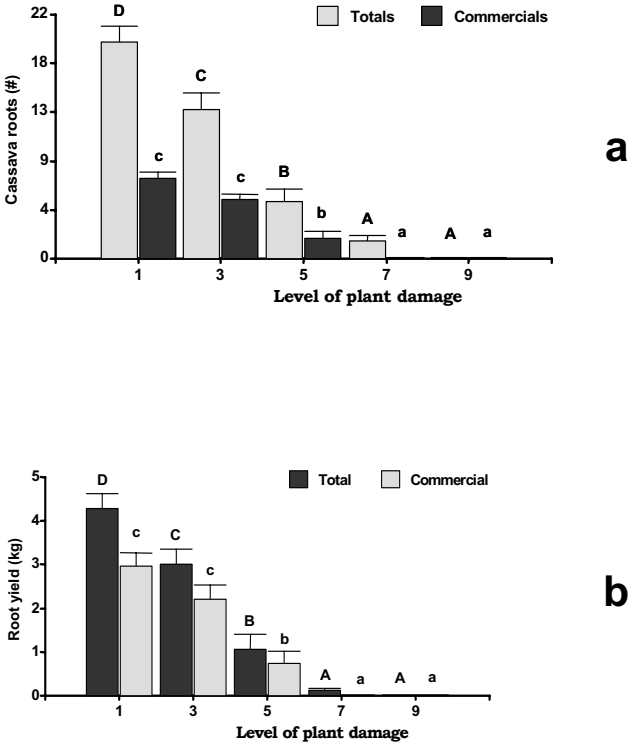

Figure 2.16.6. Evaluations to the harvest (400 dap): **a**) Number of total and commercial roots by plant; **b**) Yield in kg/plant of total and commercial roots; is shown the standard error (Tukey pÒ0,05).

Root damage due to the second white grub generation: The cassava crop's long cycle can expose it to two generations of the pest. We observed that the second-generation rhizophages developing with the maturing cassava did not damage roots, even though as many as 3 third-instar larvae were found in association with more than 100 plants in the plot.

Our findings differed from those of E. L. Melo-Molina (, unpublished data) and C. J. Herrera (unpublished data), who had detected orifices and galleries in cassava roots in Panama and Colombia. The explanation may lie with the experimental site, that is, the larvae found sufficient food in the abundant quantity of succulent rootlets, both of the cassava and neighboring plants that often grow up by harvest time in long-cycle crops. Hence, damage caused before harvest does not produce economic losses nor is it detected in leaves. We therefore recommend conserving live mulch in the crop's furrows, which would distract the insect and prevent its attacking the thickened roots.

Conclusions

- ∉ It is possible to identify levels of damage in cassava, beans, and maize within 30 days after planting and develop strategies for the control of white grubs of *Phyllophaga* spp.
- ∉ The minimal damage identified during the development of maize cause significant yield losses
- ∉ Although white grubs didn't eliminate a significant number of bean plants the minimal visible damage on leaves can diminish yield and needs means of control
- ∉ The relative economic losses are highly significant even when observed plant damage was minimal: they skirt 30% of cassava, going through 50% in maize, until reaching 60% in beans

- Healthy and slightly damaged plants obtained similar levels in terms of plant height, umber of stalks, diameter of stalk and branching of cassava
- Cassava: all levels of damage caused finally irrecoverable yield losses of roots for the fresh market
- Cassava: when damage is slight (level 3) plants continue their development producing a satisfying root weight that can be sold to for the starch market
- White grub species different to *P. menetriesi* that coincide with the last months of maize and beans prior to harvest didn't significantly effect neither vigor of the plants nor yield.
- In contrast to other authors we could not evidence that the second generation of *P. menetriesi* causes significant damage on the swollen cassava roots.
- In order to reduce yield losses in maize, cassava, and beans coincidence of planting and third larval instar of *P. menetriesi* should be avoided.
- Four weeks after planting a survey of 50 randomly selected plants per hectare should be carried out (of a density of 30,000 maize plants, 90,000 bean plants and 10,000 cassava plants per hectare). If three plants or more are associated with only one grub the farmer should initiate activities of control.
- As last strategy plants should be replaced immediately after detecting symptoms of damage and after previous elimination of the grubs. Otherwise, economic losses can oscillate between 25 and 60% even if the visual damage of the plant is minimal.

Activity 2.17. Feeding behavior of three white grub species associated with potato in the Savanna of Bogotá

Contributors: Cesar Zuluaga, Daniel Carrillo, Ilan Garzón, Miguel Serrano, Andreas Gaigl

Highlight:

- Importance of soil organic matter on pest status of three white grub species identified

Rationale

One of the most important pests in the cold zones of the Colombian Andean region are white grubs (Coleoptera: Melolonthidae) (Londoño *et al*., 2002, CORPOICA Boletín Técnico N^{o} 3, Colombia). These insects have been reported as pests on horticulture, ornamental flowers, potatoes, and pasture. The prevailing species on potato and pasture in Cundinamarca are *Clavipalpus ursinus, Heterogomphus dilaticollis, and Ancognatha scarabaeoides* (Ruiz & Posada, 1985, Rev. Colombiana de Entomología 11: 21-26). However, the pest status of these species is not clear. Scholtz (1990, Journal of Natural History 24: 1027-1066) proposed that within the Melolonthinae subfamily (i.e. C. ursinus) larvae usually feed on roots, but also on humus, or rarely, on dung; whereas larvae that belong to the Dynastinae subfamily (i.e. *A. scarabaeoides*, *H. dilaticollis*) feed on plant roots or rotting vegetable matter. In the case of A. scarabaeoides there are contradictory reports. Ruiz & Posada (1985, Revista Colombiana de Entomología 11: 21-26) claimed that this white grub feeds mostly on material in process of decomposition whereas other reports state that this grub is a serious pest on potato. Likewise, the pest status of *C.ursinus* and *H. dilaticollis* is not documented. The objective of this work was to study the feeding behavior of these three white grub species that are abundant in Savannah of Bogotá. We evaluated i) the effect of soils with different portion of SOM on the size and weight these larvae, ii) the effect of different food types on the

development of these grubs, and iii) the damage caused bythese three species on tubers, potato germs, and wood.

Materials and Methods

The complete experiment was conducted on the farm "Potosí" in the municipality of Subachoque (Cundinamarca, Colombia). Approximately 1000 white grubs of each species were collected in the field and confined in groups of 20 individuals in plastic pots (one gallon) filled with soil and germinated wheat during a period of 15 days prior to the experiments establishment. The offered food types were pieces of wood, potato tubers, or no food (control). The experimental unit consisted of one pot (one gallon) filled with sand (0% SOM) or soil (14% SOM), one food type and five larvae in second and third instar of *C. ursinus*, *H. dilaticollis*, and *A. scarabaeoides*, respectively. *A. scarabaeoides* and *A. ustulata* were present at the sites where we collected grubs. However, *A. scarabaeoides* dominated. Since it is very difficult to distinguish these larvae morphologically we included both in our tests. We weighted the white grubs every eight days and measured the diameter of the larvae every 15 days.

We estimated the consumption ("damage") on potatoes and wood using a scale from zero to four, where 0 represents no damage, 1 = 1 – 25%, 2 = 26 – 50%, 3 = 51 – 75%, and 4 = 76 – 100%.

Results and Discussion

The type of nutrition didn't significantly affect the diameter of larvae's bodies when soil was the substrate. Larvae of *Ancognatha* spp. reduced weight in all treatments. Likewise, the weight didn't significantly differ when we offered only soil, potato, or wood. However, *C. ursinus* increased weight on soil without any other food and lost weight feeding on wood and potato. *H. dilaticollis* increased weight when wood was offered and lost weight when the larvae fed only on potato or soil (Table 2.17.1). The good adaptability of *C. ursinus* and *H. dilaticollis* to soil alone and wood, respectively, attracts attention. This finding was corroborated by the low mortality (29 and 25%, respectively) of grubs in these two treatments.

When sand was the substrate none of the three white grub species found their ideal conditions. All grubs lost weight independently of the food type. Nevertheless, *Ancognatha* spp. was the species that less suffered when potatoes were offered (Table 2.17.1). Interestingly, *C. ursinus* lost more weight on pure sand and wood, indicating that this species definitely depends on fresh plant material such as potatoes (Table 2.17.2).

Table 2.17.1. Effect of three food types on weight (g) of *Ancognatha* spp., *Clavipalpus ursinus*, and *Heterogomphus dilaticollis* in soil (14% SOM). Different letters indicate significant differences between means (Tukey, $P<0.05$).

Species	No food	Wood	Potato
Ancognatha spp.	-0.057 ± 0.038 a	-0.005 ± 0.022 a	0.001 ± 0.014 a
Clavipalpus ursinus	0.062 ± 0.077 a	-0.077 ± 0.028 b	-0.034 ± 0.029 ab
Heterogomphus dilaticollis	-0.006 ± 0.003 b	0.052 ± 0.065 a	0.012 ± 0.024 ab

Table 2.17. 2. Effect of three food types on weight (g) of *Ancognatha* spp., *Clavipalpus ursinus*, and *Heterogomphus dilaticollis* in sand. Different letters indicate significant differences between means (Tukey, P<0.05).

Species	No food	Wood	Potato
Ancognatha spp.	-0.026 ± 0.102 b	-0.016 ± 0.048 b	-0.041 ± 0.139 a
Clavipalpus ursinus	-0.354 ± 0.199 a	-0.308 ± 0.222 a	-0.092 ± 0.196 a
Heterogomphus dilaticollis	-0.006 ± 0.022 a	-0.074 ± 0.019 a	-0.003 ± 0.012 a

All three species fed on potato tubers. *H. dilaticollis* caused most damage on potato in sand, whereas *C. ursinus* was the most noxious species on potato in soil with SOM. All three species fed on wood. In sand *H. dilaticollis* consumed more wood than the other species. All white grubs fed more on wood when the environment was soil.

All three species attacked in similar magnitude potato germs, this in spite of the different size of these three species; however, damage was greater in soil as substrate. This can be explained by the fact that potatoes germinated more in soil than in sand and that white grubs developed better when SOM was present. (Figure 2.17.1)

Ancognatha spp. prefers soil organic matter, however, when SOM is absent this species converts into an aggressive pest of potatoes. We conclude that this species has strong capacities to feed on potatoes under normal conditions as well as in soils of low content of SOM. This corroborates Villalobos *et al.*, 1996, Applied Soil Ecology 5: 231-246) who observed that the presence of SOM reduced feeding activity of *Costelytra zealandica* on carrots. Villalobos (1999, Journal of Sustainable Agriculture 14: 5-29) suggested that melolonthids larvae feed on a range of substrates that vary from living and dead roots of plants to other fractions of the complex pool defined as SOM or humus.

It is important to mention that in the savanna of Cundinamarca farmers cultivate intensively potatoes on plots with a slope of more than 50% deploying conventional agricultural methods like tillage promoting erosion. These conditions cause modifications of the physiochemical soil characteristics. These continued alterations are responsible of SOM reduction, forcing *Ancognatha* spp. to attack fresh tubers. Due to this insect's capability to replace easily soil organic matter with fresh tubers as food increases dramatically its pest status in this region. The increasing soil degradation during the last decades due to SOM depletion has increased root herbivory by white grubs and disrupted the presence of entomopathogens.

In spite of being the smallest larva in this experiment *C. ursinus* was the species that most damage caused on potato tubers. Moreover, it is the species that less consumed on wood in both substrates, indicating its high specialization in consumption of potatoes and little acceptance of other food alternatives.

C. ursinus is one of the most abundant species in the Savanna of Bogotá. In spite of having the capability of feeding on organic matter this grub shows a clear preference for fresh material. These results support the observations made in potato, horticulture, pasture and reports of farmers in this region that this species is the economically most important white grub.

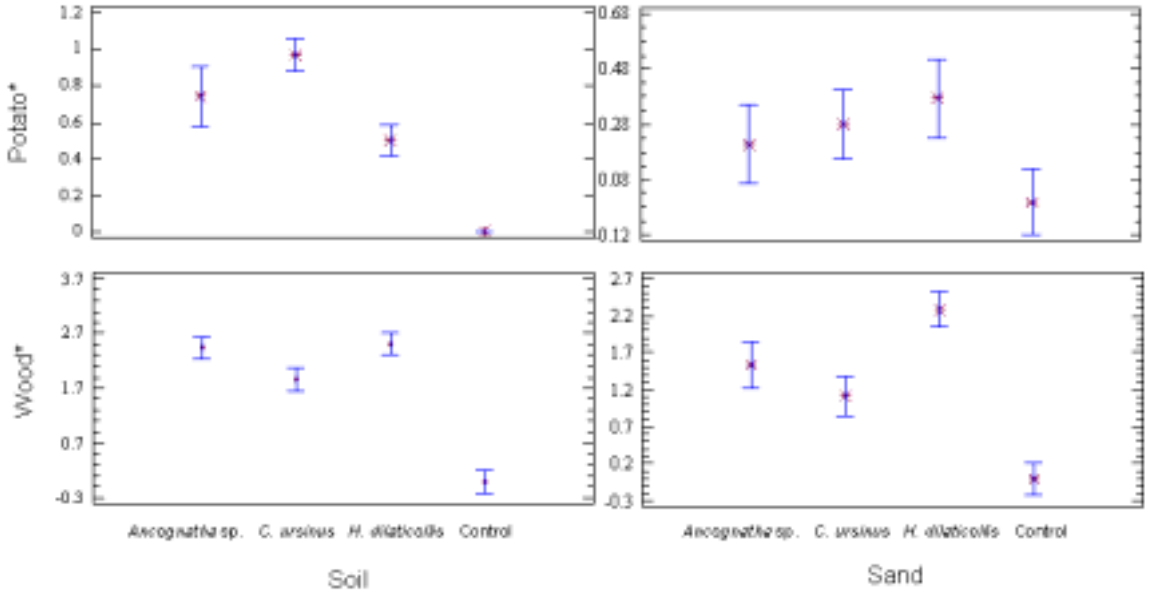

Figure 2.17.1. Feeding behavior of *Ancognatha sp., C. ursinus* and *H. dilaticollis*. Damage on potato tubers and wood in soil with SOM and sand. Means and Standard Errors (P<0.05).*measured using a scale from zero to four, where 0 represents no damage, 1 = 1 – 25%, 2 = 26 – 50%, 3 = 51 – 75%, and 4 = 76 – 100%.

H. dilaticollis was the species that most consumed on wood. This fact is corroborated by our observation that this white grub is mostly associated with trunks in decomposition in the field. We observed a pronounced preference for this type of food in spite of the capability to feed on SOM or potatoes. This explains the observation that *H. dilaticollis* caused less damage on potato than the other two species. However, when no organic matter is available this species may convert into a serious pest.

In general we want to claim that this experiment yielded highly valuable results for example the importance of SOM on the pest status of white grubs. However, other results are very difficult to explain and indicate some errors in the experimental design that should be eliminated in future works. For example, it is against any logic that larvae of *C. ursinus* weight less than they feed on potatoes and more than there is food available. It is possible that 20 larvae in one experimental unit blur the results. The variance of these 20 individuals may vary a lot. Moreover, the parameter "Diameter of Larvae" was not an appropriate indicator of the development and behavior o the grubs. It is possible that the three month lapse of observation was too short. On the other hand, weight was a good indicator of the development of the immature stages.

Conclusions and Recommendations

- ü *Ancognatha* spp. may damage potato tubers when no SOM is available
- ü The same applies to *H. dilaticollis*
- ü *C. ursinus* prefers fresh material and is a principal pest on potato and other crops. It is necessary to develop strategies of integrated control of this insect.
- ü We recommend using in future experiments only one individual per experimental unit.
- ü To evaluate effect of alternative agricultural strategies such as no tillage or crop rotation on the feeding behavior of *Ancognatha* spp. and *H. dilaticollis*.

- ü To generate programs of transferring technology for farmers that allow to identify white grub species in the zones where potatoes are cultivated and develop strategies of control

Activity 2.18. Screening *Brachiaria* genotypes for spittlebug resistance

Contributors: C. Cardona, G. Sotelo, J. W. Miles, and A. Pabón

Highlights:

- ∉ Numerous sexual hybrids (SX03, SX05) with high levels of antibiosis resistance to *Aeneolamia varia*, *A. reducta*, and *Zulia carbonaria* were identified
- ∉ High levels of antibiosis resistance to *A. varia*, *A. reducta* and *Z. carbonaria* were detected in 9 apomictic hybrids (series BR04)
- ∉ Six apomictic hybrids of the MX02 series, selected for resistance to *Prosapia simulans*, also showed resistance to *A. varia*, *A. reducta, Z. carbonaria*, and *Mahanarva trifissa*
- ∉ Six apomictic hybrids of the series BR02 and 11of the series MX02 were identified as resistant to *A. varia*, *Z. carbonaria*, *Z. pubescens*, and *M. trifissa* under field conditions

Activity 2.18.1. Greenhouse screening of *Brachiaria* accessions and hybrids for resistance to four spittlebug species

Rationale

Assessment of resistance to spittlebugs is an essential step in the process of breeding superior *Brachiaria* cultivars at CIAT. In 2005, intensive screening of selected hybrids was conducted under greenhouse and field conditions. All available genotypes were evaluated.

Materials and Methods

Screenings for resistance in the greenhouse were conducted with *Aeneolamia varia, A. reducta, Zulia carbonaria, Z. pubescens, Mahanarva trifissa* and *Prosapia simulans*. Using a new methodology (Cardona *et al*., 1999, J. Econ. Entomol. 92:490-496) test materials were usually compared with five checks fully characterized for resistance or susceptibility to *A. varia*. Plants were infested with six eggs per plant of the respective spittlebug species and the infestation was allowed to proceed without interference until all nymphs were mature (fifth instar stage) or adult emergence occurred. Plants (usually 5-10 per genotype) were scored for symptoms using a damage score scale (1, no visible damage; 5, plant dead) developed in previous years. Percentage nymph survival was calculated. Materials were selected on the basis of low damage scores (<2.0 in a 1-5 scale) and reduced percentage nymph survival (<30%). All those rated as resistant or intermediate were reconfirmed. All susceptible hybrids were discarded.

Results and Discussion

In 2005, 119 pre-selected sexual (SX03) hybrids were simultaneously screened for resistance to *A. varia*, *A. reducta*, and *Z. carbonaria*. We used five replications per hybrid per insect species. For comparison, we used five well-known checks replicated 10 times per insect species. All but one of the hybrids were

resistant to all three test species. To the extent that mean percentage nymph survival in the population did not differ from percentage survival in our most resistant check, the hybrid SX01NO/0102 (Table 2.18.1.1). These results clearly indicate that a very significant progress has been made in incorporating antibiosis resistance to all of the three test species in a relatively short period of time.

Table 2.18.1.1. Levels of resistance to three spittlebug species in 119 sexual Brachiaria hybrids and checks.

Genotype	Spittlebug species					
	Aeneolamia varia		*Aeneolamia reducta*		*Zulia carbonaria*	
	Damage scores[1]	Percentage nymph survival	Damage scores	Percentage nymph survival	Damage scores	Percentage nymph survival
BRX-44-02[2]	5.0a	86.6a	4.9a	83.3a	4.5a	79.6a
CIAT 0606[2]	4.8a	93.3a	4.4a	85.2a	4.4a	69.9a
CIAT 6294[3]	1.9bc	36.7bc	3.1b	52.4b	2.4b	50.0b
CIAT 36062[3]	2.2b	26.7bc	1.7cd	18.5c	2.2b	38.3b
CIAT 36087[4]	2.3b	48.1b	2.0c	18.3c	1.2c	1.7c
Mean 119 SX03 hybrids	1.4c	13.1c	1.2d	2.9d	1.3c	6.5c
SX01NO/0102[3]	1.2c	0c	1.1d	3.3d	1.0c	0c

[1] On a 1 – 5 damage score scale (1, no visible damage; 5, severe damage, plant killed)
[2] Susceptible check
[3] Resistant check
[4] 'Mulato 2'; commercial check.
Means of 5 reps per genotype per insect species. Means within a column followed by the same letter are not significantly different at the 5% level according to Scheffe's multiple range test for arbitrary comparisons. Each species analyzed separately.

Further proof of the rapid progress made in incorporating resistance to spittlebug was obtained when 565 new hybrids (SX05 series) were tested for resistance to three spittlebug species. As shown in Figure 2.18.1.1, 96.2%, 94,7% and 93.9% were rated as resistant to *A. varia*, *A. reducta*, and *Z. carbonaria*, respectively. 468 hybrids (82.8%) were classified as highly resistant to all three species tested. Progress was also detected when resistance reactions in two consecutive cycles were compared (Figure 2.18.1.2). It is valid to conclude that there has been a steady increase in the frequency of resistant genotypes as a result of recurrent selection through cycles.

In support of continuous breeding activities we screened a set of 141 apomictic BR04 hybrids. Most were susceptible but a handful of them showed acceptable levels of antibiosis resistance to all three tests species (Table 2.18.1.2). As in previous occasions, correlations between damage scores and percentage nymph survival were highly significant: 0.802** for *A. varia*, 0.924** for *A. reducta* and 0.840** for *Z. carbonaria*.

In 2004 we reported on varying levels of resistance to *Prosapia simulans* (one of the most important species affecting *Brachiaria* in Mexico) in 34 apomictic hybrids (coded MX). These hybrids had been pre-selected in Mexico for good adaptation and desirable agronomic characteristics. In 2005 we conducted a series of replicated tests to evaluate the resistance of these genotypes to four major species present in Colombia. Those showing multiple resistances are listed in Table 2.18.1.3.

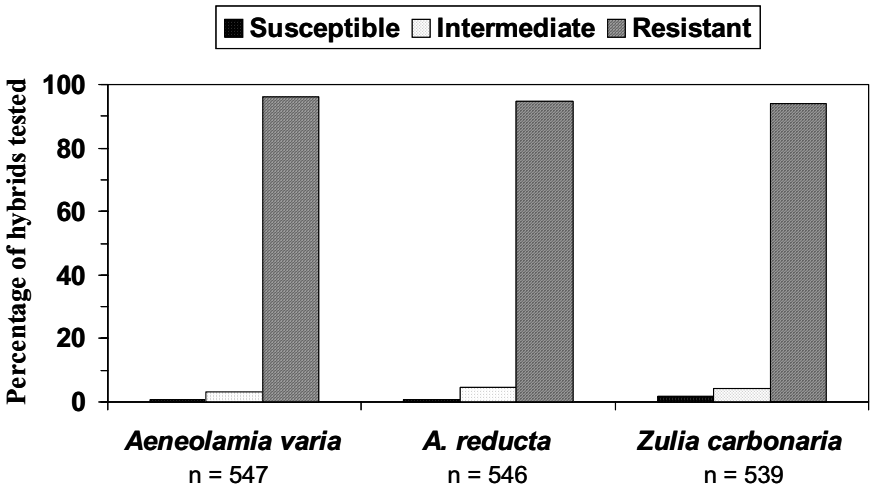

Figure 2.18.1.1. Frequency distribution of resistance reactions in a population of 565 sexual *Brachiaria* hybrids (SX05 series) tested for resistance to three major spittlebug species.

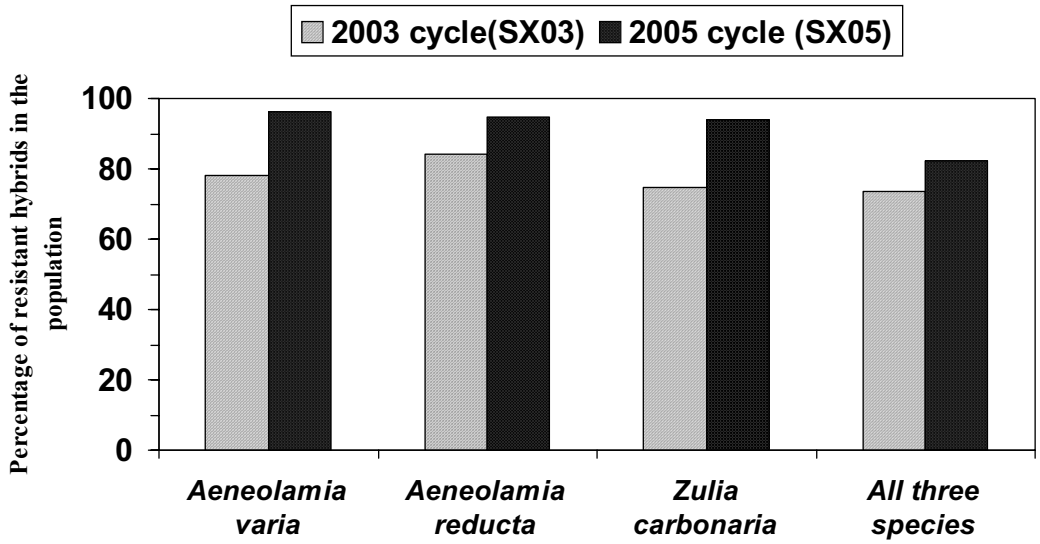

Figure 2.18.1.2. Frequency distribution of resistant reactions in two consecutive cycles of selection in *Brachiaria* for resistance to three major spittlebug species.

Table 2.18.1.2. Percentage nymph survival in selected *Brachiaria* genotypes screened for resistance to three major spittlebug species.

Genotype	Spittlebug species		
	Aeneolamia varia	***Aeneolamia reducta***	***Zulia carbonaria***
BR04NO/1751	6.7	13.3	3.3
BR04NO/1819	13.3	0	0
BR04NO/1889	10.0	-	3.3
BR04NO/2007	26.7	0	13.3
BR04NO/2405	23.3	23.3	16.7
BR04NO/2455	-	0	3.3
BR04NO/2515	33.3	6.7	10.0
BR04NO/2557	33.3	20.0	0
BR04NO/2793	-	0	16.7
BRX-44-02[1]	93.3	91.7	90.0
CIAT 0606[1]	91.7	93.3	81.7
CIAT 6294[2]	58.3	56.7	38.3
CIAT 36062[2]	12.5	13.3	25.0
CIAT 36087[3]	81.7	28.3	20.0
SX01NO/0102[2]	5.0	0	0

[1] Susceptible check
[2] Resistant check
[3] Commercial check
Means of 5 reps per genotype per species.

Table 2.18.1.3. Percentage nymph survival in selected *Brachiaria* apomictic hybrids tested for resistance to five spittlebug species. Means ± SEM of five replications per genotype.

Genotype	Spittlebug species				
	Aeneolamia varia	***Aeneolamia reducta***	***Zulia carbonaria***	***Mahanarva trifissa***	***Prosapia simulans***
MX02NO/1809	41.7 ± 9.7	45.0 ± 9.3	40.0 ± 8.7	0	16.7 ± 1.8
MX02NO/1905	25.0 ± 7.1	23.3 ± 8.3	13.3 ± 6.9	3.3 ± 2.4	3.3 ± 0.7
MX02NO/2273	3.3 ± 3.3	0	1.7 ± 1.7	13.3 ± 5.7	6.2 ± 1.9
MX02NO/2552	30.0 ± 6.5	16.7 ± 7.8	48.3 ± 10.7	0	33.3 ± 2.3
MX02NO/3056	8.3 ± 5.1	1.7 ± 1.7	13.3 ± 6.9	26.7 ± 6.2	1.7 ± 0.5
MX02NO/3213	5.0 ± 2.5	13.3 ± 7.8	43.3 ± 9.4	10.0 ± 7.1	9.2 ± 1.3
BRX-44-02[1]	88.3 ± 2.6	90.4 ± 3.7	68.3 ± 7.2	80.0 ± 5.7	68.3 ± 2.4
CIAT 0606[1]	80.0 ± 9.6	91.7 ± 3.5	75.9 ± 4.6	76.7 ± 4.7	49.9 ± 2.0
CIAT 6294[2]	38.3 ± 8.2	55.0 ± 11.1	57.0 ± 8.8	3.3 ± 2.4	6.7 ± 1.2
CIAT 36062[2]	8.3 ± 3.7	11.1 ± 7.0	30.0 ± 6.2	3.3 ± 2.4	0
SX01NO/0102[2]	5.0 ± 3.5	1.7 ± 1.7	18.3 ± 7.6	0	-
CIAT 36087[3]	63.3 ± 11.0	30.0 ± 7.8	1.7 ± 1.7	30.0 ± 8.7	1.7 ± 0.5

[1] Susceptible check
[2] Resistant check
[3] Commercial check.

Activity 2.18.2. Field screening of *Brachiaria* accessions and hybrids for resistance to four spittlebug species

Contributors: C. Cardona, G. Sotelo, and J. W. Miles

Rationale

Assessment of spittlebug resistance under natural levels of infestation in the field is very difficult due to the focal, unpredictable occurrence of the insect. This problem has been overcome since 1998 when we developed a technique that allows us to properly identify resistance under field conditions. Evaluating for resistance under field conditions is important because it allows us to reconfirm levels of resistance identified under greenhouse conditions.

Materials and Methods

Using the experimental unit described in our 1998 Annual Report (Sotelo and Cardona, 2000, Rev. Col. Entomol. 27: 17-20), the genotypes (usually 10 replicates) are initially infested in the greenhouse with an average of 10 eggs per stem. Once the infestation is well established, with all nymphs feeding on the roots, the units are transferred to the field and transplanted 10-15 days after infestation. The infestation is then allowed to proceed without interference until all nymphs have developed and adults emerge some 30-35 days thereafter. The plants are then scored for damage by means of the 1-5 visual scale utilized in greenhouse screenings. The number of stems per clump is counted before and after infestation and a tiller ratio (tillers per plant at the end of the infestation process/tillers per plant at the beginning of the infestation process) is then calculated. Using this methodology, 20 major screening trials (seven with *A. varia*, six with *Zulia carbonaria,* five with *Z. pubescens*, and two with *Mahanarva trifissa*) were conducted in Caquetá in 2005. The main purpose of these trials was to reconfirm resistance in 36 apomictic hybrids (BR02) and 34 apomictic hybrids (MX) that had been previously evaluated in Palmira under greenhouse conditions.

Results and Discussion

Using tiller ratios (the ratio between tillers per plant at the beginning of the infestation process and tillers per plant at the end of the infestation process) as the main selection criterion, we found that most of the BR02 hybrids tested were susceptible to spittlebug (Figure 2.18.2.1). A handful, listed in Table 2.18.2.1 showed a more or less acceptable level of field resistance due to the relatively high levels of antibiosis resistance present in these hybrids. The mechanism protected the plants from intense insect damage, allowing the plants to grow and lose less tillers than the susceptible checks. One of the commercial checks (CIAT 36087, 'Mulato 2') was resistant.

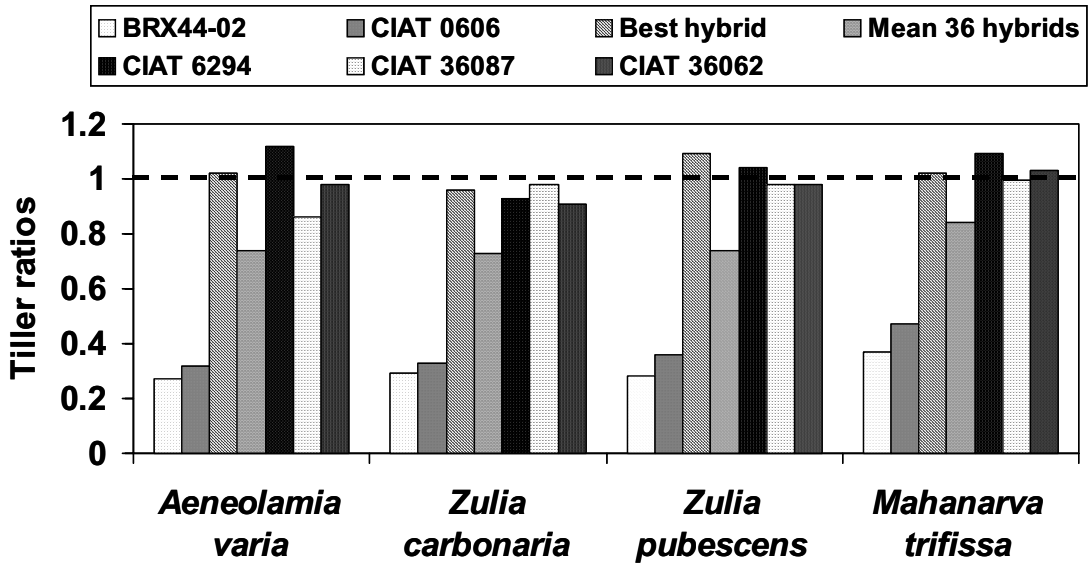

Figure 2.18.2.1. Resistance to four spittlebug species in selected *Brachiaria* apomictic (BR02) hybrids and checks tested under field conditions. The dotted line represents the cut-off point for resistance rating and selection.

Better results were obtained when 33 apomictic hybrids coded MX were tested for resistance to *A. varia*, *Z. carbonaria* and *M. trifissa*. Most of the genotypes were classified as resistant both in terms of damage scores (data not shown) and tiller ratios (Figure 2.18.2.2). The mean of selected genotypes did not differ from the mean of the resistant checks. The two commercial checks, 'Mulato' and 'Mulato 2' showed a high level of field resistance (Table 2.18.2.2).

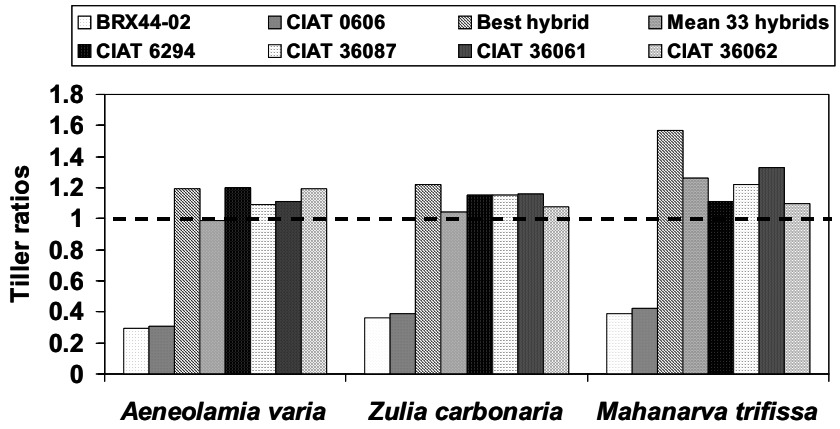

Figure 2.18.2.2. Resistance to four spittlebug species in selected *Brachiaria* apomictic (MX02) hybrids and checks tested under field conditions. The dotted line represents the cut-off point for resistance rating and selection.

Table 2.18.2.1. Tiller ratios (tillers per plant at the end of the infestation process/tillers per plant at the beginning of the infestation process) in selected *Brachiaria* genotypes tested for resistance to four spittlebug species under field conditions in Caquetá, Colombia.

Genotype	Spittlebug species			
	Aeneolamia varia	***Zulia carbonaria***	***Zulia pubescens***	***Mahanarva trifissa***
Selected hybrids				
BR02NO/1487	0.92	0.95	0.90	0.91
BR02NO/1912	0.73	0.96	0.83	0.98
BR02NO/1245	0.80	0.85	0.79	0.91
BR02NO/0638	1.01	0.65	0.69	1.02
BR02NO/0892	0.96	0.83	0.81	0.87
BR02NO/1747	0.72	0.76	1.01	0.88
Mean selected hybrids	0.86b	0.83c	0.84b	0.93b
		Resistant checks		
CIAT 6294	1.12	0.93	1.05	1.09
CIAT 36062	0.98	0.91	0.98	1.03
Mean resistant checks	1.05a	0.92b	1.01a	1.06a
		Commercial check		
CIAT 36087	0.86b	0.98a	0.98a	0.99a
		Susceptible checks		
CIAT 0606	0.32	0.33	0.36	0.47
BRX44-02	0.26	0.29	0.28	0.36
Mean susceptible checks	0.29c	0.31d	0.32c	0.41c

Means of 10 reps per genotype per species per trial; 4 trials in the case of *A. varia*, 3 trials with *Z. carbonaria* and *M. trifissa*, and 2 trials with *Z. pubescens*. Means within a column followed by the same letter are not significantly different at the 5% level according to Scheffe's multiple range test for arbitrary comparisons. Each species analyzed separately.

Table 2.18.2.2. Tiller ratios (tillers per plant at the end of the infestation process/tiller per plant at the beginning of the infestation process) in selected *Brachiaria* genotypes tested for resistance to three spittlebug species under field conditions in Caquetá, Colombia.

Genotype	Spittlebug species		
	Aeneolamia varia	***Zulia carbonaria***	***Mahanarva trifissa***
Selected hybrids			
MX02/2273	1.19	1.22	1.57
MX02/3861	1.14	1.16	1.35
MX02/3056	1.10	1.07	1.64
MX02/3213	1.14	1.10	1.38
MX02/2531	1.09	1.15	1.32
MX02/1809	1.03	1.14	1.40
MX02/1942	1.12	1.06	1.32
MX02/3567	1.06	1.14	1.20
MX02/1769	1.12	1.07	1.13
MX02/1660	1.09	1.14	1.09
MX02/3426	1.03	1.05	1.39
Mean selected hybrids	1.10a	1.11a	1.34a
Resistant checks			
CIAT 6294	1.20	1.15	1.11
CIAT 36062	1.19	1.08	1.10
Mean resistant checks	1.19a	1.11a	1.10c
Commercial checks			
CIAT 36061	1.11	1.16	1.33
CIAT 36087	1.09	1.15	1.22
Mean commercial checks	1.10a	1.15a	1.27b
Susceptible checks			
CIAT 0606	0.31	0.39	0.42
BRX44-02	0.29	0.36	0.39
Mean susceptible checks	0.30b	0.37b	0.40d

Means of 10 reps per genotype per species per trial; 3 trials with *A. varia* and *Z. carbonaria*, 2 trials with *M. trifissa*. Means within a column followed by the same letter are not significantly different at the 5% level according to Scheffe's multiple range test for arbitrary comparisons. Each species analyzed separately.

Activity 2.19. Identify host mechanisms for spittlebug resistance in *Brachiaria*

Contributors: M. F. Miller, C, Cardona, and G. Sotelo

Highlights:

- Finalized studies on the effect of host plant resistance on the demography of *Zulia carbonaria*
- Initiated studies on possible biochemical factors associated with antibiosis resistance to spittlebug
- Initiated studies on tolerance to adult feeding damage as a component of resistance to spittlebug
- Continued studies on mechanisms of resistance to *Deois incompleta, D. schah,* and *Notozulia entreriana* in Brazil and *Prosapia simulans* in Mexico. These will be reported in 2006

Activity 2.19.1. Effect of host plant resistance on the demography of *Zulia carbonaria*

Rationale

Varying levels of antibiosis resistance to nymphs of several spittlebug species have been well characterized in a number of resistant *Brachiaria* genotypes. The effects of antibiosis on the biology of nymphs have also been studied (Cardona *et al*., 2004, J. Econ. Entomol. 97:635-645). Not much was known about possible direct effects of antibiotic genotypes on the biology of adults. Even less was known about sub-lethal effects (i. e., reduced oviposition rates, reduced longevity, prolonged generation times, reduced rates of growth, etc.) on adults resulting from nymphs feeding on antibiotic genotypes. In 2004 we initiated a series of studies aimed at measuring how antibiotic genotypes may directly or indirectly (through sub-lethal effects) affect the biology of adults of *A. varia*. In 2005, similar studies were conducted with another major species, *Z. carbonaria*. We used the life-table technique, which is widely recognized as one of the most effective means of teasing apart the subtle, interrelated aspects of changes in population density. Longevity, age-specific fecundity, sex ratio and generation time can be examined and compared among treatments as they relate to the most important demographic parameter, the intrinsic rate of natural increase.

Materials and Methods

A comprehensive series of experiments aimed at determining whether antibiosis to nymphs has an adverse effect on the demography of *Z. carbonaria* were conducted. For this, 8 life tables (four fecundity, four complete) were constructed. Treatment combinations are shown in Table 2.19.1.1. For each of these treatments we established cohorts of 105 pairs of spittlebug and the fate and reproductive rate of individuals were recorded until death occurred. From these data the following life-table statistics were derived: net reproductive rate (R_o) [net contribution per female to the next generation]; mean generation time (T) [mean time span between the birth of individuals of a generation and that of the next generation]; doubling time (D) [time span necessary to double the initial population]; finite rate of population increase (ə) [multiplication factor of the original population at each time period]; and intrinsic rate of natural increase (r_m) [innate capacity of the population to increase in numbers]. Life-table statistics were analyzed using the SAS program based on jackknife estimates of demographic parameters (Maia *et al*., 2000, J. Econ. Entomol. 93: 511-518). Other variables recorded were sex ratios, percentage egg fertility and adult dry weights. These data were submitted to analysis of variance and when the *F* test was significant, we performed mean separation by LSD.

Results and Discussion

Sub-lethal effects of resistance on the demography of Zulia carbonaria: The resistant genotype SX01NO/0102 caused significant effects on the demography of *Z. carbonaria*. In general, rearing of nymphs of *Z. carbonaria* on the resistant genotype had a deleterious effect on the weight of resulting males and on the number and fertility of eggs laid per female (Table 2.19.1.2).

Table 2.19.1.1. Treatment combinations to study possible sub-lethal effects of high levels of nymphal antibiosis on adults of *Zulia carbonaria*.

Nymphs reared on:	**Resulting adults feeding on:**	**Null hypothesis**
CIAT 0654[a]	CIAT 0654	Absolute check
CIAT 0654	SX01NO/0102	A genotype that is highly antibiotic to nymphs does not affect adults
SX01NO/0102	CIAT 0654	High antibiosis to nymphs does not affect resulting adults
SX01NO/0102	SX01NO/0102	High antibiosis to nymphs does not affect resulting adults even when these are feeding on a highly antibiotic genotype

[a] CIAT 0654 is a highly susceptible accession; SX01NO/0102 (a resistant hybrid) possesses high levels of antibiosis resistance to nymphs of *Z. carbonaria*.

Table 2.19.1.2. Life history parameters of *Zulia carbonaria* as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes.

Treatment[a]		**Adult dry weight ($g \times 10^{-3}$)**		**Eggs per female**	**Percentage egg fertility**
Nymphs reared on:	**Resulting adults feeding on:**	Females	Males		
CIAT 0654 (S)	CIAT 0654 (S)	1.52a	0.81a	451.4a	97.4a
CIAT 0654 (S)	SX01NO/0102 (R)	1.48b	0.78ab	440.0a	96.8a
SX01NO/0102 (R)	CIAT 0654 (S)	1.47b	0.75bc	353.7b	88.1b
SX01NO/0102 (R)	SX01NO/0102 (R)	1.43c	0.73c	286.9c	85.2c

[a] S, susceptible; R, resistant.
Within a column, means followed by the same letter are not significantly different at the 5% level by LSD.

Age-specific survival and age-specific fecundity curves for *Z. carbonaria* adults are presented in Figure 2.19.1.1. Mean survival times for the four treatment combinations did not differ at the 5% level, meaning that there was not a major impact of nymphal antibiosis on the survival of resulting males or females. On the contrary, rearing of the insect on the resistant genotype SX01NO/0102 did have a pronounced effect on the ability of resulting females to lay eggs. Independently of the food substrate used to feed the adults, females obtained from rearing the nymphs on the resistant genotype laid fewer eggs, for a slightly shorter period of time, than those obtained from rearing the insect on the susceptible genotype. This can be interpreted as a sub-lethal effect of nymphal antibiosis on the reproductive capacity of the insect.

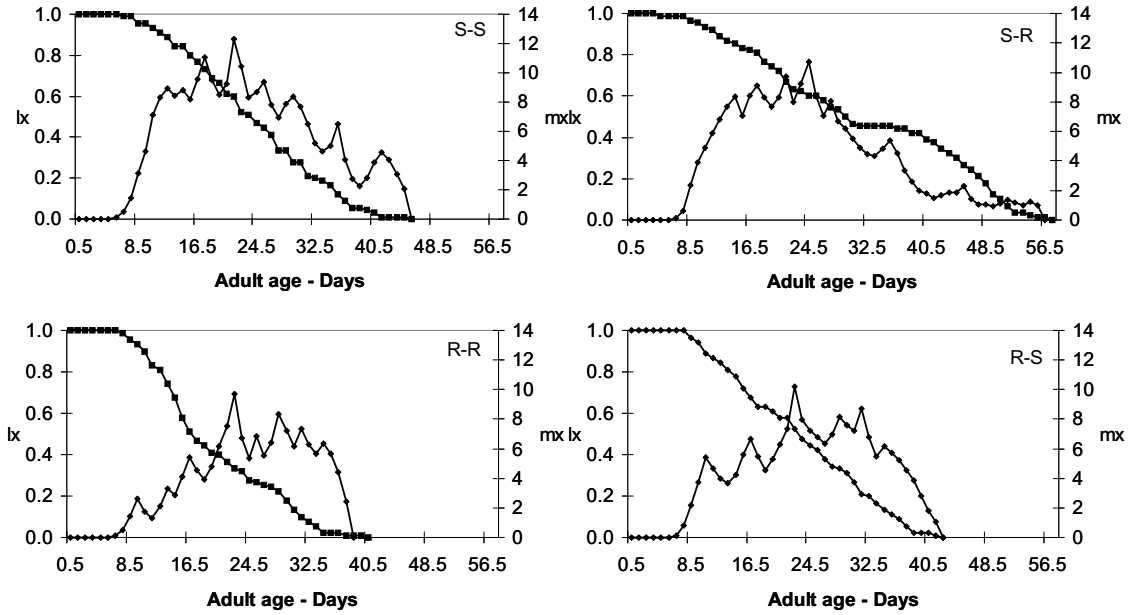

Figure 2.19.1.1. Age-specific survival (l_x) (y) and age-specific fecundity (m_x) (D) curves for adults of *Zulia carbonaria* as affected by all possible combinations of food substrate for adults and nymphs. First initial in letter combinations indicates the food substrate for nymphs followed by the initial for the food substrate for resulting adults. S, susceptible genotype (CIAT 0654); R, resistant genotype (SX01NO/0102).

All demographic parameters of *Z. carbonaria* adults were significantly affected by the antibiotic effect of SX01NO/0102 on the nymphs (Table 2.19.1.3). Females originating from nymphs reared on the resistant genotype had lower net reproductive rates, lower intrinsic rates of natural increase, and lower finite rates of increase than those obtained from rearing the insect on the susceptible genotype. We conclude that antibiosis to nymphs in the resistant *Brachiaria* hybrid SX01NO/0102 causes significant sub-lethal effects on the reproductive biology of resulting adults.

Total effects of resistance on the demography of Zulia carbonaria: To measure the total impact of antibiosis resistance on the demography of *Z. carbonaria*, we took into account the rates of immature mortality caused by both the resistant and the susceptible genotypes. Age-specific survival curves for nymphs and adults, as well as age-specific fecundity curves for *Z. carbonaria* adults are presented in Figure 2.19.1.2. The antibiosis to nymphs present in the resistant genotype SX01NO/0102 had a significant deleterious effect on the biology of the insect, which reflected in very high levels of immature mortality. As a result, survival curves were very low as compared to those obtained with the susceptible genotype. Rearing of the insect on the resistant genotype caused a delay in the emergence of adults. Antibiosis also had a significant effect on the ability of resulting females to lay eggs. Independently of the food substrate used to feed the adults, females obtained from rearing the nymphs on the resistant genotype laid less eggs than those obtained from rearing the insect on the susceptible genotype.

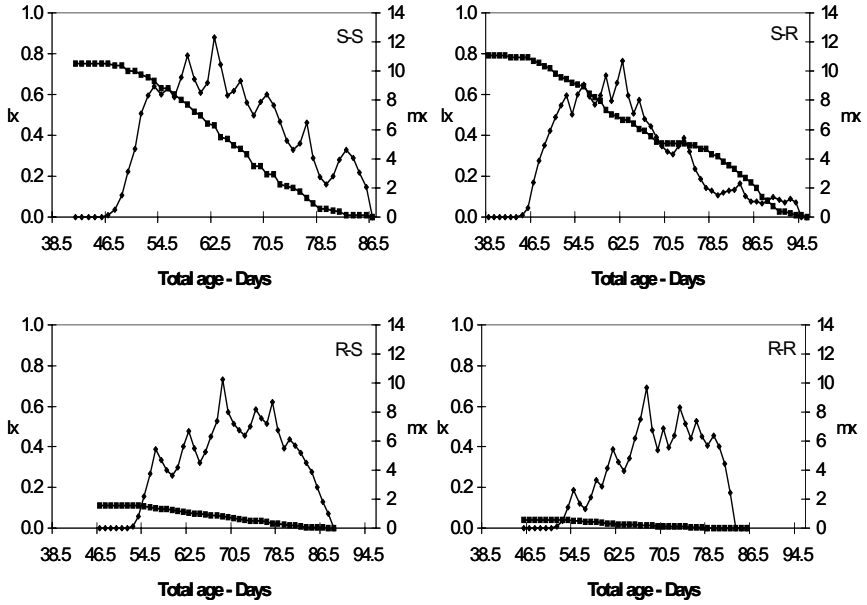

Figure 2.19.1.2 Age-specific survival (l_x) (y′) and age-specific fecundity (m_x) (Dž curves for *Zulia carbonaria* as affected by all possible combinations of food substrate for adults and nymphs. First initial in letter combinations indicates the food substrate for nymphs followed by the initial for the food substrate for resulting adults. S, susceptible genotype (CIAT 0654); R, resistant genotype (SX01NO/0102).

As a result of high immature mortality and sub-lethal effects on resulting adults, all demographic statistics of the *Z. carbonaria* population tested were significantly affected by the antibiosis present in SX01NO/0102 . Populations derived from the resistant genotype had lower net reproductive rates, lower intrinsic rates of natural increase, lower finite rates of increase and longer generation times than those obtained from rearing the insect on the susceptible genotype.

Table 2.19.1.3. Fecundity life-table statistics for *Zulia carbonaria* adults as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes.

Treatment[a]		**Demographic parameters**		
Nymphs reared on:	**Resulting adults feeding on:**	**Net reproductive rate (R_o)**	**Intrinsic rate of natural increase (r_m)**	**Finite rate of increase (ə)**
CIAT 0654 (S)	CIAT 0654 (S)	229,8a	0.295a	1.344a
CIAT 0654 (S)	SX01NO/0102 (R)	230.1a	0.267a	1.306b
SX01NO/0102 (R)	CIAT 0654 (S)	184.3b	0.260b	1.297b
SX01NO/0102 (R)	SX01NO/0102 (R)	140.6c	0.248c	1.282c

[a] S, susceptible; R, resistant

Within a column, means followed by the same letter are not significantly different at the 5% level by LSD Jackknife estimates of the intrinsic rate of increase (per capita rate of population growth).

The finite rate of increase is a parameter that describes deleterious effects on a given population. It is defined as a multiplication factor of the original population at each time period. The decimal part of the finite rate of increase corresponds to the daily rate of increase expressed as a percentage. This means that populations reared on the susceptible genotype would grow by 8% whereas those on the resistant genotype would grow by 2.3-4.2% (Table 2.19.1.4). We conclude that high immature mortality caused by the resistant *Brachiaria* hybrid SX01NO/0102 and sub-lethal effects of antibiosis on resulting adults have a very major impact on the demography of *Z. carbonaria*.

Table 2.19.1.4. Life-table statistics for *Zulia carbonaria* as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes

Treatment[a]		Demographic parameters				
Nymphs reared on:	**Adults feeding on:**	**Net reproductive rate (R_o)**	**Intrinsic rate of natural increase (r_m)**	**Mean generation time (T)**	**Doubling time (Dt)**	**Finite rate of increase (λ)**
CIAT 0654 (S)	CIAT 0654 (S)	172.3a	0.077a	66.7b	9.0c	1.080a
CIAT 0654 (S)	SX01NO/0102 (R)	181.8a	0.071a	66.5b	8.9c	1.081a
SX01NO/0102 (R)	CIAT 0654 (S)	20.3b	0.041b	73.8a	17.0b	1.042b
SX01NO/0102 (R)	SX01NO/0102 (R)	5.6c	0.023c	74.8a	29.9a	1.023c

[a] S, susceptible; R, resistant

Within a column, means followed by the same letter are not significantly different at the 5% level by LSD Jackknife estimates of the intrinsic rate of increase (per capita rate of population growth).

Activity. 2.19.2. Studies on possible biochemical factors associated with antibiosis resistance to spittlebug

Contributors: C. Cardona, G. Sotelo, J. Miles (CIAT) and Brent Brodbeck (University of Florida)

Rationale

As stated before, high levels of antibiosis resistance to nymphs of several spittlebug species have been well characterized in numerous resistant *Brachiaria* genotypes. Identification of the biochemical basis of spittlebug resistance, and development of rapid and precise biochemical assays for resistance would provide a valuable addition to breeding efforts to introgress spittlebug resistance into adapted *Brachiaria* germplasm. Scientists at the University of Florida have long proposed that changes in xylem-feeders development may be related to differences in xylem nutrient profiles (i. e. subtle differences in xylem nutrients may result in varying developmental success of the insect). To test this possibility we approached Drs. Brent V. Brodbeck and Peter C. Andersen who kindly accepted our request to analyze xylem samples taken from resistant and susceptible *Brachiaria* genotypes.

Materials and Methods

We used an array of genotypes well characterized for resistance or susceptibility to *A. varia*: 18 sexual hybrids (SX03), two susceptible checks (accessions BRX44-02 and CIAT 0606) and three resistant checks (accessions CIAT 36062, CIAT 6294 and the sexual hybrid SX01NO/0102). Plants were grown in large pots in the greenhouse (24^{o} C, 75% R.H.) and infested with 100 mature eggs each. Infestation was then allowed to proceed without interference. When nymphs reached the fourth instar stage, the plants were cut off at approx. 3 cm from the soil surface. Several stems of approx. 4- to 5-mm diameter were wrapped with tape to increase their effective diameter. The 8-mm interior diameter nozzle of a plastic, disposable syringe was fitted over the entire cut end of the stem wrapped with tape to make a tight connection. Taping externally further sealed the union of the nozzle of the syringe and the cut stem. Suction was applied by withdrawing the syringe plunger, which was held in the withdrawn position until the desired volume of liquid accumulated within the syringe. Xylem samples thus obtained were immediately frozen and shipped to the University of Florida where they were analyzed for contents of 19 different amino acids.

Results and Discussion

There was not a significant correlation between amino acid contents and resistance ratings based on percentage nymph survival. In spite of these disappointing preliminary results, we intend to continue this line of research using a small grant from the USAID-University linkage fund.

Activity 2.19.3. Studies on tolerance to adult feeding damage as a component of resistance to spittlebug

Contributors: F. López, C. Cardona, and G. Sotelo

Rationale

Our studies have clearly identified nymphal antibiosis as the main mechanism of resistance to several different species of spittlebug in many different *Brachiaria* genotypes. In fact, we have also been able to document rapid progress in the incorporation of antibiosis resistance to nymphs in sexual and apomictic hybrids developed through a recurrent selection-breeding scheme (Miles et al., 2005, accepted for publication in Crop Science). Given that adults can be as damaging as the nymphs, it is widely accepted that antibiosis to nymphs should be combined with an acceptable level of tolerance to adult feeding damage. However, nothing is known about mechanisms of resistance to adult feeding damage in *Brachiaria*. For this reason, and for the first time, in 2005 we initiated a series of studies aimed at characterizing tolerance as a possible component of resistance to spittlebug.

Materials and Methods

To study tolerance to adult feeding we initially compared the response of the susceptible accession CIAT 0654 and the resistant hybrid SX01NO/0102 to increasing levels of infestation with adults of *A. varia*. Thirty-day old plants of CIAT 0654 and SX01NO/0102 were exposed to 0, 2, 3, 5, 7, 9, 12, and 15 adults per plant. The 16 host genotype-infestation level treatment combinations were randomly assigned to single-plant experimental units with 10 replications per treatment combination. Plants were infested with neonate adults and the infestation was allowed to proceed until all adults died. Percentage adult survival was calculated. Damage scores in a 1-5 visual damage score scale were taken 5 and 10 days after

infestation. To measure chlorophyll loss as a result of adult feeding, we used a SPAD-502 chlorophyll meter 5 and 10 days after infestation. Four representative readings per plant were taken and their averages were recorded. SPAD index values were then calculated with respect to the uninfested checks. At the end of the trial, when all insects had died, plants were cut at soil level and dried in an oven at 40^{o} C. Percentage biomass losses were calculated with respect to the uninfested checks. Damage scores and percentage biomass losses were used to calculate functional plan loss indices.

Results and Discussion

Adult survival was not affected by the genotype when plants were infested with 2, 3 or 5 adults per plant. At higher infestation levels (7, 9, 12, and 15 adults per plant) adult survival on the susceptible genotype was significantly lower possibly due to depletion of food and increased competition among insects. This means that a 5-6 level of infestation could be used in future studies. SX01NO/0102 plants suffered significantly less damage than susceptible (CIAT 0654) plants at all levels of infestation (Figure 2.19.3.1).

At all levels of infestation, SX01NO/0102 plants suffered significantly less damage (expressed as percentage chlorophyll loss and percentage biomass loss) than susceptible CIAT 0654 plants (Figure 2.19.3.2). Significant correlations were found between damage scores and percentage chlorophyll losses ($r = 0.858$; $P < 0.001$), between damage scores and percentage biomass losses (0.473; $P < 0.001$) and between percentage chlorophyll losses and percentage biomass losses ($r = 0.891$; $P < 0.001$) indicating that damage scores are useful in predicting losses and that SPAD units are useful in measuring insect damage.

Furthermore, when a Functional Plant Loss Index (combining damage scores and percentage biomass losses) was calculated, we found that at all levels of infestation losses were highest for the susceptible genotype CIAT 0654 (Figure 2.19.3.3) Since no obvious signs of antibiosis to adults were found in this experiment, we interpret lower damage scores, lower chlorophyll and biomass losses, and lower functional plant losses as the expression of tolerance to adult feeding damage in the resistant genotype. Further results on this line of research, aimed at developing a mass screening procedure for adult spittlebug damage, will be reported in 2006.

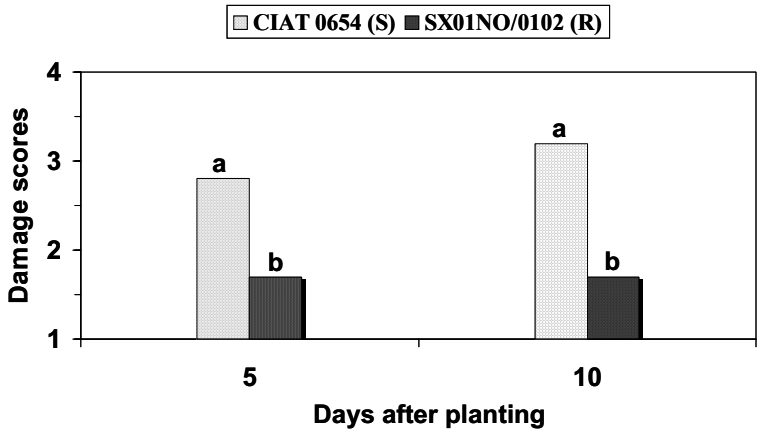

Figure 2.19.3.1. Response of susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes to attack by adults of *Aeneolamia varia*. Means of eight levels of infestation. For each scoring date, bars with the same letter do not differ at the 5% level of significance by LSD.

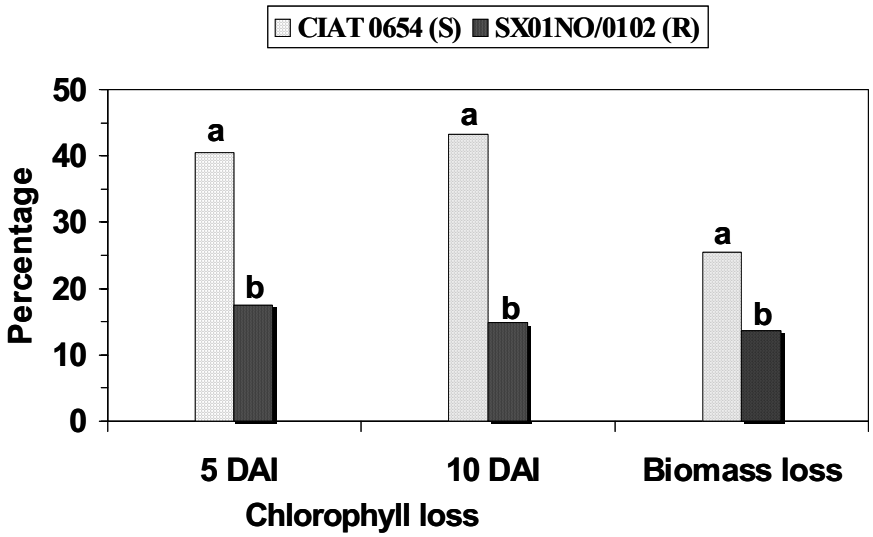

Figure 2.19.3.2. Chlorophyll and biomass losses due to adult *Aeneolamia varia* feeding on susceptible (CIAT 0654) or resistant SX01NO/0102 *Brachiaria* genotypes. DAI, days after infestation. Means of eight levels of infestation. For each variable, bars with the same letter do not differ at the 5% level of significance by LSD.

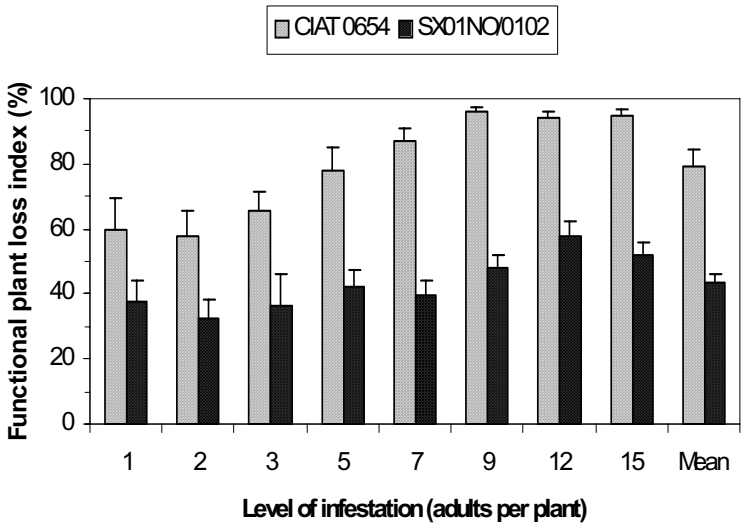

Figure 2.19.3.3. Functional plant loss indices (percentage) for susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes exposed to eight levels of infestation with adults of *Aeneolamia varia*.

Activity 2.20. Validating thermotherapy of stem cuttings, and use of Lonlife® and *Trichoderma* for managing cassava diseases in the Eastern Plains and Cauca, Colombia

Contributors: Elizabeth Álvarez, Germán Llano, John Loke, Juan Fernando Mejía,Víctor Montaña, Wilson Gaitán, and Bolívar Muñoz

Highlight:

- Cassava bacterial blight was successfully controlled under field conditions by treating stem cuttings with hot water, Meta, Colombia.

Rationale

Bacterial blight, *Phytophthora* root rots, and superelongation disease are widespread and cause high losses in important cassava-producing regions in Colombia. Several ecological control practices, like thermotherapy and the use of biocontrol agents, have been evaluated recently for managing root rots in cassava. In this report, we discuss the progress made for each of the project's objectives, which are as follows: (1) with farmer participation, to adjust and validate strategies of integrated management of the constraining diseases found in each region; (2) to improve, through training, farmers' knowledge of integrated disease management; (3) to contribute to the technical and commercial development of the biological inputs needed to implement integrated management of the economically most important diseases of cassava; (4) to evaluate the economic sustainability of the proposal in terms of its effects on competitiveness in employment, income, and internal rate of return; and (5) to evaluate the financial sustainability of the proposal in terms of financing this project's products for introduction into the production sector.

Materials and Methods

Six commercial plots of cassava were established in five municipalities, two in each of the departments of Cauca and Meta, and one in Casanare. The aims were:

- To evaluate the performance of several promising cassava varieties under the conditions of two agroecological areas: the Eastern Plains and Andean Region (Department of Cauca; Figure 2.20.1)

- To validate the effect of treating stakes with Lonlife®, a product of low toxicity and derived from seeds of citrus fruits

- To validate the performance of the fungi *Trichoderma viride* Persoon and *T. harzianum*, which attack soil pathogens and have shown to control several species of *Phytophthora*, causal agents of root rots

Figure 2.20.1. Planting cassava plots in the Department of Cauca, Colombia.
Eastern Plains: Cantaclaro (Puerto López).

Three semicommercial plots were established on the farms "La Vega" (Aguazul, Casanare) and "Cantaclaro" (Puerto López, Meta), and CORPOICA's research farm "La Libertad", located in Villavicencio (Meta), to evaluate the performance of four promising cassava varieties and the effect of treating stakes with Lonlife® and of inoculating them with *T. viride* and *T. harzianum.*

We planted 0.5 ha with the varieties La Reina, Vergara, and CM 4574-7, and treated the stakes and soil as described below. For comparison, 9 ha were also planted with the same varieties under farmer management. Planting was on the furrow ridges.

Treatments. Good quality stakes were selected from productive healthy plants. They were treated as follows:

a Stakes were immersed for 10 min in a solution with Lonlife® and the insecticide Roxion® (dimethoate), each at 2 cc/L.

b Farmers immersed stakes for 10 min in a solution of copper oxychloride (at 3 g/L) and Roxion® (at 2 cc/L). This treatment was used as check.

The fungus *T. viride* strain CIAT-14PDA-4 -an antagonist and plant growth stimulator- was applied directly to the soil around planted cassava stakes, once at 1 month after planting and again at 3 months. The product AgroGuard® (containing *T. harzianum*) were added, each at 0.5 g/L. For the fungus, this was the equivalent of 2.5×10^8 spores/L. The farmer also used the product Bioderma® (containing *T. harzianum*).

Results and Discussion

Evaluations of germination, vigor, and incidence of disease were conducted by the technicians handling the crop. These evaluations will serve to define crop management practices, which are urgently needed as the area planted to the crop expands in response to demand for fuel-alcohol production from cassava.

The variety La Reina had a higher rate of germination than had either Vergara or CM 4574-7. Vergara showed improved germination with Lonlife®. Although CM 4574-7 had the lowest rate, it was similar to the two evaluated stake treatments (Table 2.20.1).

'CM 4574-7' showed no symptoms of either superelongation disease (SED) or cassava bacterial blight (CBB), while 'La Reina' was the most affected by both diseases. The technicians regard CM 4574-7 as the variety that so far shows the best performance.

Although it is too early to observe significant differences of effect between the two *Trichoderma* strains, CM 4574-7 plants were more vigorous under the AgroGuard® treatment, whereas the other two varieties showed no change in vigor between the strains.

La Vega (Aguazul): Two cassava varieties, La Reina and ICA Catumare, were planted on 0.35 ha and the following treatments were carried out for the stakes and soil:

Treatments: Good quality stakes were selected from productive and healthy plants.

a. Stakes were immersed in a solution of Lonlife® at 2 cc/L for 10 min.

b. Stakes were immersed for 10 min in a solution of copper oxychloride (at 3 g/L) and Lorsban® (at 3 cc/L).

c. Stakes received no treatment.

d. Two applications of each fungus strain were used to inoculate the soil around the plants at 1 and 3 months after planting. The inoculum was either the fungus *T. viride* strain CIAT-14PDA-4 or the product AgroGuard® (*T. harzianum*), each at 0.5 g/L, which was equivalent to 2.5×10^8 spores/L.

As shown in Table 2.20.2, the germination rate of the two varieties was more than 96%, except for La Reina without treatment, when germination was 84.19%. Some of the seed treated with Lonlife® (no *Trichoderma*) germinated at a rate of 88.36% because the soil had not been adequately prepared. Inoculation with *Trichoderma* had no relationship with germination because it was applied 30 days after planting.

Table 2.20.1. Rates of germination (G) of cassava stakes, vigor, and incidence of superelongation disease (SED) and cassava bacterial blight (CBB) according to different treatments of stakes and inoculations of the soil with the fungi *Trichoderma viride* and *T. harzianum*, Farm "Cantaclaro", Puerto López, Department of Meta, Colombia.

Variety and treatment	G (%)	Vigor	Incidence (%) SED	Incidence (%) CBB
CM 4574-7				
Lonlife®/*Trichoderma* strain, AgroGuard®	37	Excellent	0	0
Lonlife®/*Trichoderma* strain, Bioderma®	48	Good	0	0
Lonlife®/*Trichoderma* strain, CIAT	51	Good	0	0
Chemical treatment	46	Excellent	0	0
Average germination rate	45.5			
La Reina				
Lonlife®/*Trichoderma* strain, AgroGuard®	58	Average	10	20
Lonlife®/*Trichoderma* strain, Bioderma®	49	Average	15	40
Lonlife®/*Trichoderma* strain, CIAT	61	Average	10	50
Chemical treatment	60	Average	10	25
Average germination rate	57.0			
Vergara				
Lonlife®/*Trichoderma* strain, AgroGuard®	46	Good	3	0
Lonlife®/*Trichoderma* strain, Bioderma®	71	Good	5	0
Lonlife®/*Trichoderma* strain, CIAT	50	Good	5	0
Chemical treatment	40	Good	10	15
Average germination rate	51.8			

La Libertad (Villavicencio): Three cassava varieties, La Reina, Vergara, and CM 4574-7 were planted on 0.5 ha and the following treatments were carried out:

Treatments:

a. Healthy stakes were immersed for 10 min in a solution of Lonlife® at 2 cc/L.

b. Healthy stakes were immersed for 10 min in a solution of copper oxychloride (at 3 g/L) and malathion (at 2 cc/L).

c. Stakes infected with the bacterium *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) were placed in hot water (49 °C) for 49 min.

d. Stakes infected with *Xam* and given no treatment.

e. Two applications of each fungus strain were used to inoculate the soil around the plants at 1 and 3 months after planting. The inoculum was either the fungus *T. viride* strain CIAT-14PDA-4 or the product AgroGuard® (*T. harzianum*), each at 0.5 g/L, which was equivalent to 2.5×10^8 spores/L.

The germination rate for all treatments was very low, with a maximum of 57.5%. Using healthy plant stakes, the variety with the lowest rate was Vergara (36.3%). Treatment with Lonlife® slightly improved germination rates for varieties La Reina and CM 4574-7 (Table 2.20.3).

Table 2.20.2. Germination rate of two cassava varieties receiving different stake treatments, Farm "La Vega", Aguazul, Department of Casanare, Colombia.

Variety and treatment	Germination rate (%)
La Reina	
Lonlife®–no *Trichoderma*	88.36
Lonlife®–*Trichoderma* strain CIAT	96.86
Lonlife®–*Trichoderma* strain AgroGuard®	96.86
Chemical–no *Trichoderma*	97.47
Chemical–*Trichoderma* strain CIAT	99.24
Chemical–*Trichoderma* strain AgroGuard®	98.99
No treatment	84.19
ICA Catumare	
Lonlife®–no *Trichoderma*	99.02
Lonlife®–*Trichoderma* strain CIAT	98.62
Lonlife®–*Trichoderma* strain AgroGuard®	96.66
Chemical–*Trichoderma* strain CIAT	98.74
Chemical–*Trichoderma* strain AgroGuard®	98.99
No treatment	96.19

Table 2.20.3. Germination of stakes and dead plants from stakes infected with *Xanthomonas axonopodis* pv. *manihotis* receiving different stake treatments, Farm "La Libertad", Villavicencio, Department of Meta, Colombia.

Variety and treatment[a]	Germination (%)	Dead plants (%)[b]
La Reina		
Healthy stakes with Lonlife®	57.5	0.5
Healthy stakes with chemical treatment	54.3	0.2
Stakes with CBB, no treatment	35.4	22.9
Thick stakes with CBB, heat therapy	39.1	20.3
Thin stakes with CBB, heat therapy	23.1	3.1
CM 4574-7		
Healthy stakes with Lonlife®	54.6	0.0
Healthy stakes with chemical treatment	49.6	0.2
Vergara		
Healthy stakes with Lonlife®	29.6	0.6
Healthy stakes with chemical treatment	36.3	0.2

a. Healthy stakes were selected from plants with no symptoms of cassava bacterial blight (CBB).
b. From CBB-infected stakes.

For stakes from plants infected with CBB, the heat treatment reduced disease incidence to 3.1% in plants that developed from thin stakes, whereas incidence for thick stakes was 20.3% and the check (no treatment) 22.9%. Normally, germination is reduced when thin stakes are used. These results suggest that thick stakes should receive heat treatment over a longer period to guarantee cleaning of the material. However, farmers should be recommended to select more strictly for healthy plant stakes.

Department of Cauca: La María: Together with the Local Agricultural Research Committee (CIAL, its Spanish acronym) "La María", in the Municipality of Piendamó, we established two plots on the farms of Lilia Rojas and Arbey Agredo. The goal was to evaluate the performance of three promising cassava varieties and the effect of treating stakes with Lonlife® and of inoculating the soil with *T. viride* and *T. harzianum.* Evaluations were made with the active participation of the farmers forming the CIAL.

Varieties: These were SM 707-17, SM 1498-4, and SM 1495-5.
Treatments: Good quality stakes were selected from productive and healthy plants.

a. Stakes were immersed for 10 min in a solution of Lonlife® at 2 cc/L.

b. Stakes were immersed for 10 min in a solution of *Trichoderma* strain AgroGuard® (Live Systems Technology S.A.) or of strain CIAT (0.5 g/L, equivalent to 2.5×10^8 spores/L).

c. Stakes received no treatment.

d. One application of each fungus strain was used to inoculate the soil around the plants at 2 months after planting. The inoculum was either the fungus *T. viride* strain CIAT-14PDA-4 or the product AgroGuard® (containing *T. harzianum*) at 0.5 g/L each.

The farmers evaluated the cassava according to their own criteria, indicating good, regular, or bad results in terms of germination, vigor, and vegetative development. They also ranked the varieties according to their preference (Table 2.20.4). On day 25 after planting, germination was evaluated. The rate for variety SM 1495-5 was lower than for the other two varieties. The farmers did not observe differences among treated and untreated stakes with regard to germination rate, but observed better development of plants from treated stakes.

In an evaluation made jointly with the farmers, when the crop was 55 days old, the best variety was SM 707-17, followed by SM 1498-4, and then SM 1495-5. The plot at Lilia Rojas' farm showed better development. According to the farmers, the cassava crop in Arbey Agredo's plot did not develop well, produced less, and had more rot. Stakes treated with Lonlife® had higher germination rates and greater vigor. Differences were yet to be observed between the two *Trichoderma* treatments.

San Bosco: We established a plot together with the CIAL "San Bosco", in the Village District of Mondomo, Municipality of Santander de Quilichao. The goal was to evaluate the performance of two promising cassava varieties and the effect of treating stakes with Lonlife® or of inoculating the soil with *T. viride* and *T. harzianum.* The evaluations were made with the active participation of the farmers forming the CIAL.

Varieties: These were SM 707-17 and CM 7436-7.
Treatments: Good quality stakes were selected from productive and healthy plants.

a. Stakes were immersed for 10 min in a solution of Lonlife® at 2 cc/L.

b. Stakes were immersed for 10 min in a solution containing *Trichoderma* strain AgroGuard® (Live Systems Technology S.A.) or strain CIAT at 0.5 g/L each, the equivalent of 2.5×10^8 spores/L).

c. Stakes received no treatment.

d. Two applications of each fungus strain were used to inoculate the soil around the plants at 1 and 3 months after planting. The inoculum was either the fungus *T. viride* strain CIAT-14PDA-4 or the product AgroGuard® (containing *T. harzianum*) at 0.5 g/L each.

The farmers evaluated the cassava according to their own criteria, indicating good, regular, or bad results in terms of germination, vigor, and vegetative development (Table 2.20.5). On day 35 after planting, germination was evaluated together with the farmers from the CIAL. Variety SM 707-17 was observed as having the best development. At that time, clear differences could not yet be observed among stake treatments. The farmers used the criteria germination, development, plant vigor, and quantity and uniformity of foliage. No differences were yet observed between the two strains of *Trichoderma* or with respect to the check, which received no treatment.

Table 2.20.4. Participatory evaluation of varieties and treatment of cassava stakes, CIAL "La María", Piendamó, Department of Cauca, Colombia.

		Development evaluation[a]			Preference rank of variety[b]		
Treatment	**Variety**	**Mario**	**Jeimer**	**Gerardo**	**Mario**	**Jeimer**	**Gerardo**
Arbey Agredo's plot							
Lonlife®	SM 1495-5	R	R	R	3	3	3
Lonlife®	SM 1498-4	G	G	G	2	2	2
Lonlife®	SM 707-17	G	R	G	1	1	1
Check (no treatment)	SM 1495-5	R	R	R	3	3	3
Check (no treatment)	SM 1498-4	R	G	R	2	2	2
Check (no treatment)	SM 707-17	R	G	G	1	1	1
Lilia Rojas' plot							
Lonlife®	SM 1495-5	G	VG	VG	3	2	3
Lonlife®	SM 1498-4	G	G	G	2	3	2
Lonlife®	SM 707-17	VG	VG	VG	1	1	1
Check (no treatment)	SM 1495-5	R	G	G	3	2	3
Check (no treatment)	SM 1498-4	R	R	R	2	3	2
Check (no treatment)	SM 707-17	G	G	G	1	1	1

a. Three farmers, whose names appear in the column headings, evaluated the plots. VG = very good germination and development; G = good germination, development, and architecture; R = regular germination and development.

b. The three farmers ranked the cassava varieties according to their germination, development, and vigor, where 1 = the best and 3 = the poorest variety.

Table 2.20.5. Participatory evaluation of treated cassava stakes, CIAL "San Bosco", Santander de Quilichao, Department of Cauca, Colombia.

	Development evaluation[a]	
Variety and treatment	**Bernardino**	**Jobel**
CM 7436-7		
Lonlife®	G	G
Lonlife® + *Trichoderma* strain AgroGuard®	G	G
Lonlife® + *Trichoderma* strain CIAT	G	G
Trichoderma strain AgroGuard®	G	VG
Trichoderma strain CIAT	G	VG
No treatment	G	G
SM 707-17		
Lonlife®	VG	VG
Lonlife® + *Trichoderma* cepa AgroGuard®	G	VG
Lonlife® + *Trichoderma* strain CIAT	VG	G
Trichoderma strain AgroGuard®	G	G
Trichoderma strain CIAT	G	G
No treatment	G	G

a. Two farmers, whose names appear in the column headings, evaluated the plots. VG = very good germination and development; G = good germination, development, and architecture.

Activity 2.21. Evaluation of *Brachiaria* hybrids for resistance to *Rhizoctonia solani* under field conditions in Caqueta

Contributors: Gustavo Segura, William Mera, Ximena Bonilla, John Miles, Segenet Kelemu

Highlight:

- The resistant accession (*B. brizantha* 16320) and four Brachiaria hybrids showed high levels of resistance to Rhizoctonia foliar blight under field conditions.

Rationale

Rhizoctonia foliar blight, caused by *Rhizoctonia solani* Kühn, is a disease of increasing importance on a number of crops. The disease can be very destructive when environmental conditions are particularly conducive (high relative humidity, dense foliar growth, high nitrogen fertilization, and extended wet periods).

Rhizoctonia solani is the most widely known species of *Rhizoctonia* with a wide host range. In nature *R. solani* reproduces mainly asexually and exists as vegetative mycelia and/or dense sclerotia. These sclerotia can survive in soil and on plant debris for several years, and can germinate and produce hyphae that can infect a wide range of host plants. The pathogen primarily infects below ground plant parts in a number of plant species, but can also infect above ground plant parts such as pods, fruits, and leaves and stems as is the case with *Brachiaria*. In *Brachiaria*, infected leaves first appear water-soaked, then

darken, and finally turn to a light brown color. Lesions may coalesce quickly during periods of prolonged leaf wetness and temperatures between 21 and 32 ∀C.

Disease management through the use of host resistance, when available, remains to be the most practical and environmentally friendly strategy. Differences in reaction to *R. solani* exist in genotypes of *Brachiaria.* The ability to uniformly induce disease and measure resistance accurately is crucial in a breeding program for developing resistant cultivars. The objectives of this study are to: 1) artificially inoculate and induce uniform disease development in selected *Brachiaria* genotypes generated by CIAT's tropical forages project, 2) accurately measure resistance and identify resistant materials among these *Brachiaria* genotypes.

Materials and Methods

Plant materials: 137 *Brachiaria* genotypes with BR04 series and provided by the breeding program were planted in the field at Macagual ICA/CORPOICA Research Station in Florencia, Caquetá. CIAT 16320, CIAT 36061 and CIAT 36087 were included as controls. The field location is highly conducive to the development of the disease, with mean annual relative humidity of 84 %, an average temperature of 25.5^{o}C and an annual rainfall of 3793 mm.

Field layout, artificial inoculations and disease evaluations: Six plants (that were generated from the same mother plant) of each of the *Brachiaria* genotypes were transplanted from a CIAT glasshouse to the field site in Caquetá. The space between plants was 80 cm, and 1 m between blocks. The entries were replicated 3 times in a randomized complete block design. Plants were inoculated one month after transplanting by placing 0.7 g dry sclerotia of *R. solani* isolate 36061 on the soil surface at the base of each plant. Plants were evaluated for disease reaction 15, 20, 34 and 38 days after inoculations, using the 0 – 5 (0 = no visible infection; 5 = 20 -100% of the aerial portion of the plant infected) scale that we developed earlier and reported in the 2004 Annual Report.

Results and Discussion

Disease symptoms developed fully in susceptible genotypes 10-15 days after inoculations. Plants were evaluated for disease reaction 15, 20, 34 and 38 days after inoculations. There was a high degree of correlation in disease evaluation data among the various evaluation dates (Table 2.21.1).

Table 2.21.1. Correlations between disease reaction data collected at various days after inoculations using Pearson's Correlation

Days	15	20	34	38
15	1.00	0.82	0.69	0.60
20	0.82	1.00	0.85	0.78
34	0.69	0.85	1.00	0.94
38	0.60	0.78	0.94	1.00

The resistant control CIAT 16320 was consistently evaluated at scale 2. Four genotypes, BR04-2577, BR04-2557, BR04-2983, and BR04-1214 were evaluated at an average between 2.0 and 2.5. Twenty-four others, 1685, 1950, 1963, 3077, 1119, 1252, 1347, 1349, 1824, 1886, 1896, 2060, 2200, 2201, 2265, 3025, 3207, CIAT 36087, 1928, 1941, 2040, 2069, 3066, 3217, scored with an average rating scale of 3.0-3.3 (in the rating scale, this corresponds to a 6% - 9% overall plant tissue damage). The remaining 111 materials, 2429, 2518, 2539, 3051, 3175, 1061, 1219, 1796, 1845, 2774, 2793, 2874, 3214, 1021, 1073,

1141, 1819, 2404, 2405, 2457, 2475, 2515, 2532, 2841, 2940, 3056, 222, 1265, 1311, 1358, 1377, 1592, 1648, 1956, 2110, 2118, 2128, 2179, 2208, 2275, 2403, 2938, 2987, 3069, 3119, 3221, 36061, 1081, 1097, 1113, 1197, 1281, 1296, 1503, 1633, 1697, 2007, 2226, 2235, 2389, 2414, 2969, 1018, 1026, 1060, 1273, 1309, 1360, 1374, 1570, 1629, 1883, 1889, 2093, 2163, 2290, 2338, 2346, 2670, 2681, 2792, 2833, 2849, 2872, 2954, 3068, 3128, 1003, 1058, 1494, 1754, 2109, 2285, 2344, 1428, 1596, 1601, 1751, 1846, 1900, 2156, 2166, 2455, 2863, 2871, 3058, 3134, 2360, 2396, 1552, 3130, scored between 3.5- 5.0. Figure 2.21.1 shows a graphical representation of the results using data from representative genotypes from each of these three groups.

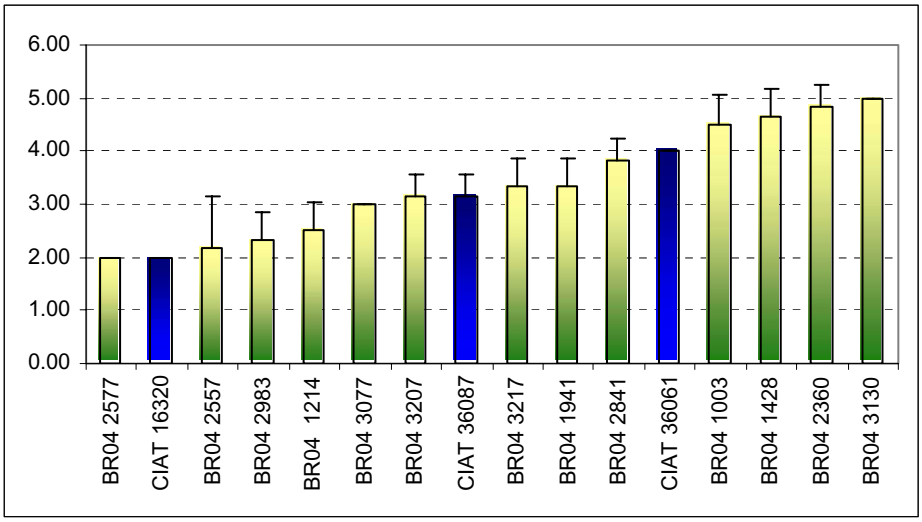

Figure 2.21.1. Ratings of *Brachiaria* genotypes for foliar blight disease reaction on a 1-5 scale 38 days after inoculations with sclerotia of *Rhizoctonia solani* under field conditions, Caquetá, Colombia. Bars indicate standard deviation.

The disease evaluation data taken 38 days after inoculations represented well-developed disease symptoms. The resistant control CIAT 16320 and the four genotypes BR04- 2577, BR04-2557, BR04-2983, BR04-1214 showed less than 6% overall plant tissue damage, and thus, a high-level of resistance (Figure 2.21.2). The second group of 24 genotypes including CIAT 36087 listed above still had an acceptable level of resistance. All the plants in this trial will be maintained in the field to further observe the level of disease at an extended period of time.

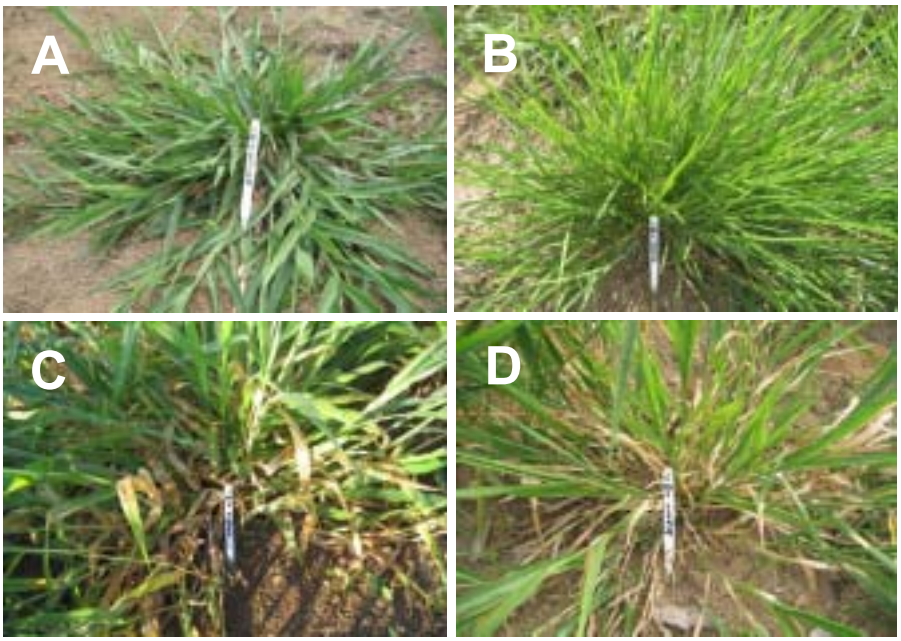

Figure 2.21.2. Rhizoctonia foliar blight disease symptoms 34 days after inoculations under field conditions in Caquetá, Colombia. **A**: BR04-1214; **B**: BR04-2577: **C**: CIAT 36061; **D**: BR04-1754

Activity 2.22. Endophyte transformation and use as gene delivery system

Contributors: Javier Abello, Celsa Garcia (Univ. Nacional, Bogota) and Segenet Kelemu

Rationale

Acremonium implicatum is an endophytic fungus that forms symbiotic association with species of *Brachiaria*. The green fluorescent protein (GFP) gene, isolated from the jellyfish *Aequorea Victoria*, or its derivatives have been expressed in a wide array of organisms including plants and microbes. The practical implication of seed transmission of endophytes in *Brachiaria* is significant: once associated with the plant, the fungus can perpetuate itself through seed, especially in apomictic genotypes of *Brachiaria*, for as long as seed storage conditions do not diminish the survival of the fungus. Several *Brachiaria* hybrids obtained from CIAT's forage breeding program were shown to harbor *A. implicatum*. Therefore, we may be able to exploit this association and its high seed transmission (Dongyi and Kelemu, 2004, Plant Disease 88:1252-1254) by using a transgenic *A. implicatum* as a vehicle for production and delivery of gene products of agronomic interest into the host plant to enhance protective benefits and other traits, and thus improve livestock production. In addition, we want to exploit the qualities of GFP as a reporter and study the interactions between *A. implicatum* and its host *Brachiaria*.

This work describes the establishment of a transformation protocol and expression of the green fluorescent protein (GFP) gene in an isolate of *Acremonium implicatum*. In this study, we used a GFP expression vector, pSK1019, to transform *A. implicatum*.

Materials and Methods

Plasmid: Plasmid pSK1019 kindly provided by Dr. Seogchan Kang of the Department of Plant Pathology, University of Pennsylvania, was used. The plasmid contains the *egfp* gene under the promoter of a gene encoding glyceraldehyde-3-phosphate dehydrogenase (GPD) isolated from *Cochliobolus heterostrophus*. It also contains a hygromycin B resistance gene hph, controlled by the *Aspergillus nidulans* trpC promoter, as well as the Kan gene for kanamycin resistance. Hygromycin B, is an aminoglycosidic antibiotic produced by *Streptomyces hygroscopicus*, and is used for the selection and maintenance of prokaryotic and eukaryotic cells transformed with the hph gene. Vector pCAMBIA 1300 that has CaMV 35S promoter, Kan gene and hph gene was used as control.

Preparation of A. implicatum cells: A. implicatum isolate 6780-201v isolated from *Brachiaria brizantha* CIAT 6780 was used for transformation of its conidia or mycelia. The fungus was grown on YMG agar (D- glucose 4,0g; malt extract 10,0g; yeast extract 4,0g; agar 10,0g; 1L distilled water) medium for 8 days and incubated at 28ºC. Conidia were collected in a solution of 0.15M NaCl and cleaned by passing through a Whatman #1 filter paper. The conidia were then suspended in YMG liquid medium and incubated with shaking (250 rpm) for 4 hours at 28ºC, in order to induce conidial germination. Subsequently, the conidia were collected by filtration and re-suspended in an induction medium IM+AS, (in 1 litre of distilled water: 2.05g K_2HPO_4; 1.45g KH_2PO_4; 0.15g NaCl; 0.5g Mg_2SO_4 .7H_2O; 0.07g $CaCl_2$ .2H_2O; 0.0025g $Fe_2SO4.7H_20$; 0.5g $(NH_4)_2SO_4$; 10 mM D-glucose; 0.5%glycerol; 40mM MES [2-n-morppholino ethanesulfonic acid]; 200σM acetosyringone at a concentration of $1x10^6$ conidia/ml. To obtain mycelia for transformation, the protocol describe above was used, but the incubation was extended to 48 hours instead of only 4 hours, and the concentration was adjusted to OD_{600}= 0.35 in IM+AS.

Transformation of A. implicatum: The transformation protocol was based on the methods described by Mullins *et al.* (2001, Phytopathology 91:173-180) for transformation of the pathogenic fungus *Fusarium oxysporum*. Some modifications were introduced. *A. tumefaciens* strains AGL1 and LBA4404 were transformed with vectors pSK1019 or pCAMBIA 1300 using methods described by Den Dulk-Ras and Hooykaas (1995, Methods Mol Biol. 55: 63- 72.). The transformed bacteria were grown in TYNG medium (for 1L medium: 10.0 g tryptone, 5.0g NaCl, 5.0 g yeast extract, 0.5g $MgSO_4 \notin 7H_2O$, pH 7.5) supplemented with kanamycin [100 σg/ml] and incubated at 28°C in the dark for 16 hours to optical density (OD_{600}) of 0.75. This bacterial cell concentration was subsequently diluted with the induction medium IM+AS to OD_{600}= 0.1 and further incubated for 4 hours to induce virulence genes. Once the incubations was completed, the bacterial cell concentration was adjusted to OD_{600}= 0.2. The *A. implicatum* preparations described above and these *A. tumefaciens* transformant cells were mixed together in equal volumes. Two hundred-σl of each mixture was placed on a 0.45-σm-pore-size, 45-mm diameter nitrocellulose membrane (Whatman, Hillsboro, OR), and plated on IM+AS agar medium (glucose content reduced to 5mM). These mixtures were incubated for 48, 60 and 72 hours. The membranes were subsequently transferred to Petri plates containing YMG agar media containing hygromycin B (100 σg/ml), and cefotaxime (500 σM), and incubated further at 28 °C. Putative transformant *A. implicatum* cells became apparent on the selection media after 5 days of incubation. Control *A. implicatum* cells were treated the same way except that they were co-cultivated with strains of *A. tumefaciens* that were not transformed with the plasmid vectors.

PCR amplifications: DNA isolated (Kelemu *et al*., 2003, Molecular Plant Pathology 4:115-118) from putative transformant bacteria and fungus as well as control ones was analyzed using the polymerase chain reaction (PCR) using primers with sequences of *egfp* and/or *hph*.
[primers glGFP3 (5`-GCCGAGCTCAGATCTCACTTGTACAGCT CGT-3`) and glGFP5 (5`-GCCGGAATTCATGAACAAGGG CGAGGAACTG-3`)]and hph122U (5`-TTCGATGTAGGAGGGCGTGGAT-3`) and hph725L (5`-CGCGTCTGCTGCTCCATACAAG-3`)].

Amplifications were carried out in a Programable Thermal Controller (MJ Research, Inc) programmed to 35 cycles comprised of 45 seconds denaturation step at 94°C (4 minutes for the first cycle), followed by 1 min at 60°C, and primer extension for 1.5 minute (10 minutes in the final cycle) at 72°C. The amplification products were separated by electrophoresis in a 1.0% agarose gel (Bio-Rad Laboratories), stained with ethidium bromide, and photographed under UV lighting.

Southern blot analysis: The DNA of 19 randomly selected putative *A. implicatum* transformants were analyzed using Southern blot analysis. The hygromycin B resistance gene hph was used as a probe. Southern hybridization was carried out using standard procedures described in Sambrook *et al.,* (1989, Cold spring Harbour Laboratory Press, Cold Spring Harbour, NY). Labeling and detection were carried out using Dig-high prime DNA labeling and detection Kit II (Roche Applied Science)

Microscope examination: The putative GFP-expressing transformants were examined under a LEICA fluorescence microscope fitted with a Leica D filter with an excitation range between 355 and 425 nm, and an H3 filter with an excitation range between 420 and 490 nm.

Plant inoculations: Brachiaria seedlings were inoculated with a few selected *A. implicatum* transformants using the method described earlier (Kelemu *et al.,* 2001, Canadian Journal of Microbiology 47:55-62).

Results and Discusión

The endophytic fungus *A. implicatum* was successfully transformed with e*gfp* (enhanced green fluorescent protein) gene. Enhanced color variants [ECFP (cyan), EGFP (green), EYFP (yellow)] have been generated through mutagenesis and these are some of the most widely used reporters in biological research. They can be used as tags to track proteins in living cells, as reporters to monitor promoter activity, and as labels to visualize specific tissues, whole cells or sub-cellular organelles. They are useful for monitoring gene expression and protein localization.

The GFP protein (27 kDa) is a spontaneously fluorescent protein that absorbs light at maxima of 395 and 475 nm and emits at a maximum of 508 nm. This protein is a success as a reporter because it requires only UV or blue light and oxygen, but requires no cofactors or substrates as many other reporters do for visualization.

In 2004, we reported the successful transformation of *A. implicatum* with GFP gene in vectors pWGFP20 and pCT74, although the green fluorescence emitting appeared to be weak, and thus the need for more work to be done in order to get tranformants with a more pronounced emission. We report this year on work of a successful transformation of the fungus with intense emissions.

The protocol that we developed for the transformation of this endophytic fungus is based on the protocol described by Mullins *et al.* (2001, Phytopathology 91:173-180)) for the pathogenic fungus *Fusarium oxysporum*. However, modifications were needed for a successful transformation of *A. implicatum*. For example, *A. implicatum* is a slow growing fungus, and thus the recommended concentration of cefotaxime (200 µM) to inhibit the growth of *A. tumefaciens*, was not sufficient enough to prevent bacterial growth from impeding the growth of *A. implicatum*. Results from the experiments we conducted indicated that cefotaxime concentrations at 500 σM was sufficient to inhibit the growth of *A. tumefaciens* while allowing *A. implicatum* putative transformants to grow on selection media. Introducing TYNG medium instead of MM [for 1L medium: 2.05g K_2HPO_4, 1.45g KH_2PO_4, 0.15g NaCl, 0.5g $MgSO_4 \notin 7H_2O$, 0.07g $CaCl_2 \notin 2H_2O$, 0.0025g FeSO4 $\notin 7H_20$, 0.5g $(NH_4)_2SO_4$] has reduced the time needed to reach the required bacterial concentration (OD_{600} = 0.75) from 48 hours to only 16. In addition, the TYNG medium eliminated the cell aggregation problem we encountered with the growth of *A. tumefaciens* (particularly with strain LBA4404) in MM and that interfered with the transformation process.

A better transformation efficiency was obtained with *A. tumefaciens* strain AGL-1 (Table 2.22.1; Figure 2.22.1). Although *A. implicatum* transformants containing either pSK1019 (*trpC* promoter) or pCAMBIA 1300 (CaMV35S promoter) were obtained, a significantly higher number of transformants were obtained with pSK1019 (Table 2.22.1). However, this suggests that the CaMV35S promoter can function in *A. implicatum* although at a much lower efficiency. The colony size of transformants in both cases is similar with an average size of 19-mm after 12 days of incubation at 28^{O}C on the selection medium.

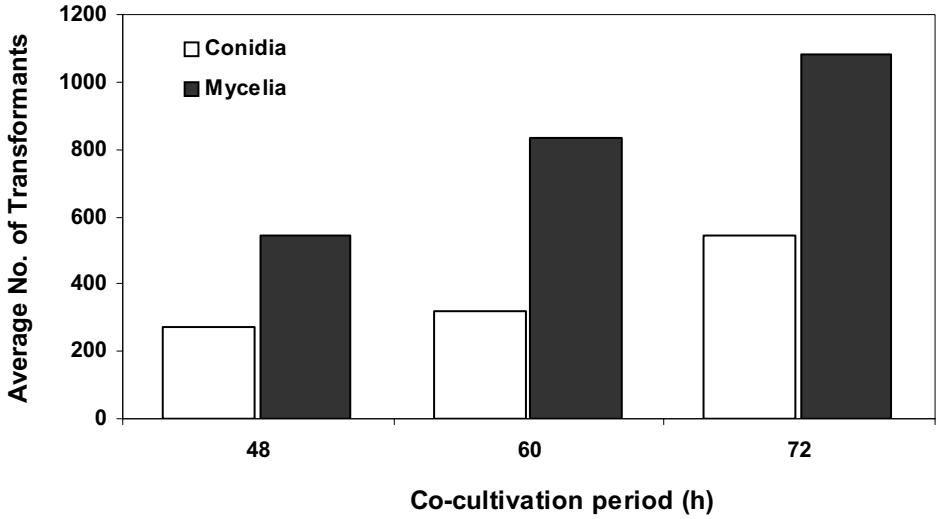

Figure 2.22.1. The effect of *Agrobacterium tumefaciens* strain AGL-1 and *Acremonium implicatum* co-cultivation period on transformation efficiency. The *A. tumefaciens* strain contains plasmid pSK1019 that has enhanced green fluorescent protein *(egfp)* gene under the promoter of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GPD) isolated from the fungus *Cochliobolus heterostrophus*. It also contains a hygromycin B resistance gene hph, controlled by the *Aspergillus nidulans trp*C promoter. The data presented are the average of three plates per treatment.

The results indicate that the transformation efficiency is directly influenced by the length of the co-cultivation (*A. tumefaciens* and *A. implicatum*) of period (Table 2.22.1, Figure 2.22.1). As the co-cultivation period increased from 48 h to 72 h, the efficiency increased from 542 transformant colonies to 1084 in the case of mycelial transformation protocol; and from 271 to 542 for conidial transformation (Table 2.22.1, Figure 2.22.1). Similar results have been reported for transformation of *Magnaporte grisea* (Rho *et al.*, 2001, Mol. Cells 3: 407-411) and *F. oxysporum*. The efficiency of transformation also differed depending on whether we used mycelia or conidia for transformation (Figure 2.22.1). The best and optimum transformation results were obtained with *A. tumefaciens* strain AGL-1, plasmid pSK1019 under the control of *trp*C promoter either with mycelial or conidial transformation. However, mycelial transformation consistently generated significantly higher number of transformants than when conidia were used to transform (Figure 2.22.1).

A. tumefaciens-mediated transformation has long been applied to transfer foreign genes to a wide-range of plants. In recent years, this has also been used to transform a wide range of fungi allowing efficient genetic manipulations of the recipient organisms. The presence of acetosyringone is important for successful *A. tumefaciens*-mediated transformation.

Table 2.22.1. Putative *Acremonium implicatum* transformant colonies per Petri dish of selection medium.

***A. tumefaciens* strain**		**AGL-1**				**LBA4404**			
Promoter		***trpC***		**CaMV35S**		***trpC***		**CaMV35S**	
Recipient fungal structure		M*	C	M	C	M	C	M	C
Co-cultivation period (Hours)	48	542	271	1,7	2	0,7	0,3	0	0
	60	836	318	1,3	3,3	1	1,0	0	0
	72	1084	542	0	0	1,3	1,7	0	0

* M = Mycelia, C = Conidia. The values represent the average number of transformants between three plates.

The putative *A. implicatum* transformants selected on hygromycin B containing agar media were further examined using fluorescence microscope, PCR and Southern blot analysis. The PCR method allowed us to quickly examine and further confirm putative transformants that have been selected on antibiotic selection media (Figures 2.22.2 and 2.22.3). To determine the copy number of the transferred T-DNA, genomic DNA from19 randomly picked transformants from each experimental condition was digested with *Hind*III and anlalyzed with Southern blot. The results exhibited genomes with inserts ranging from a single insert to 5 inserts (data not shown), while the negative control, untransformed *A. implicatum*, showed no hybridization. No correlation existed between the average copy number of T-DNA per genome and the co-cultivation period, the mycelial or conidial transformation or other variables introduced in the experiments.

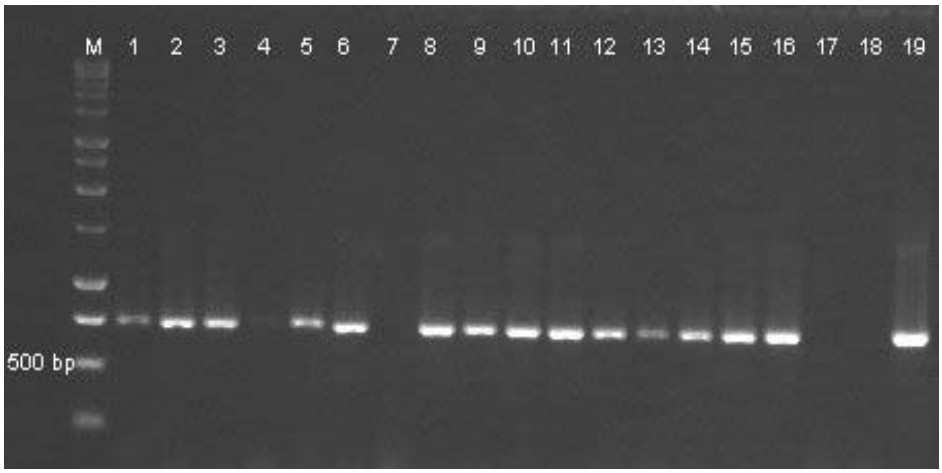

Figure 2.22.2. Polymerase chain reaction (PCR) amplifications, with primer specific for sequences of hygromycin B resistance gene (hph), of template DNA isolated from *Acremonium implicatum* transformants. Lanes M = molecular marker; template DNA from: 1 = conidia transformed with pSK1019 in *A. tumefaciens* strain LB4404 co-cultivated for 72 hours, and maintained without antibiotic selection pressure; 2, 3 = conidia transformed with pSK1019 in *A. tumefaciens* strain AGL-1 co-cultivated for 48 hours, 60 hours and maintained without or with antibiotic selection pressure, respectively; 4 = conidia transformed with pCAMBIA1300 in strain AGL-1 co-cultivated for 72 hours, and maintained without antibiotic selection pressure; 5 = mycelia transformed with pSK1019 in strain LB4404 co-cultivated for 60 hours, and maintained without antibiotic selection pressure; 6 = mycelia transformed with pSK1019 in strain AGL-1 co-cultivated for 72 hours, and maintained with antibiotic selection pressure; 7 = negative control (water); 8-11 = mycelia transformed with pSK1019 in strain AGL-1 co-cultivated for 48 hours (lanes 8, 9, and 10) and 72 hours, and maintained with antibiotic selection pressure or without it (lane 10); 12 = conidia transformed with pSK1019 in strain LB4404 co-cultivated for 72 hours, and maintained with antibiotic selection pressure; 13 = conidia transformed with pSK1019 in strain LB4404 co-cultivated for 72 hours, and maintained without antibiotic selection pressure for 4 generations; 14-16 = conidia or mycelia (lane 16) transformed with pSK1019 in strain AGL-1 co-cultivated for 48 hours and 60 hours (lane 16) and maintained with antibiotic selection pressure; 17, 18 = negative controls *Phaeoisariopsis griseola* and *A. implicatum* strain 6780 201V, respectively ; 19, 20 = positive controls pSK1019 and pCAMBIA1300, respectively.

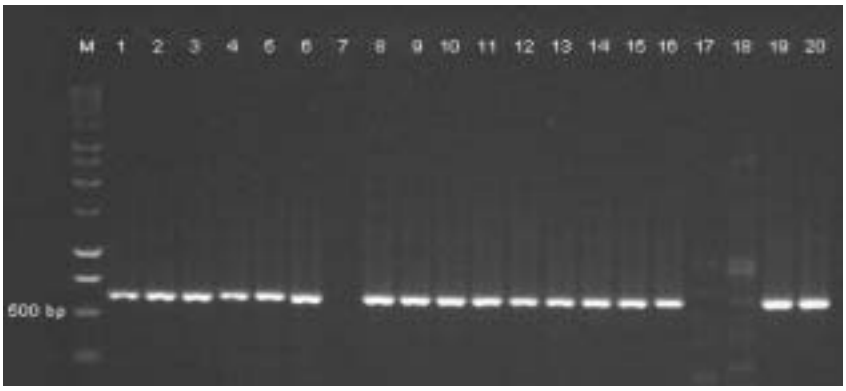

Figure 2.22.3. Polymerase chain reaction (PCR) amplifications, with primer specific for sequences of enhanced green fluorescent protein (*egfp*) gene, of template DNA isolated from *Acremonium implicatum* transformants. Lanes M = molecular marker; 1 = conidia transformed with pSK1019 in *A. tumefaciens* strain LB4404 co-cultivated for 72 hours, and maintained without antibiotic selection pressure; 2, 3 = conidia transformed with pSK1019 in *A. tumefaciens* strain AGL-1 co-cultivated for 48 hours, 60 hours and maintained without or with antibiotic selection pressure, respectively; 4 = conidia transformed with pCAMBIA1300 in strain AGL-1 co-cultivated for 72 hours, and maintained without antibiotic selection pressure; 5 = mycelia transformed with pSK1019 in strain LB4404 co-cultivated for 60 hours, and maintained without antibiotic selection pressure; 6 = mycelia transformed with pSK1019 in strain AGL-1 co-cultivated for 72 hours, and maintained with antibiotic selection pressure; 7 = negative control (water); 8-11 = mycelia transformed with pSK1019 in strain AGL-1 co-cultivated for 48 hours (lanes 8, 9, and 10) and 72 hours (lane 11), and maintained with antibiotic selection pressure or without it (lane 10); 12 = conidia transformed with pSK1019 in strain LB4404 co-cultivated for 72 hours, and maintained with antibiotic selection pressure; 13 = conidia transformed with pSK1019 in strain LB4404 co-cultivated for 72 hours, and maintained without antibiotic selection pressure for 4 generations; 14-16 = conidia or mycelia (lane 16) transformed with pSK1019 in strain AGL-1 co-cultivated for 48 hours and 60 hours (lane 16) and maintained with antibiotic selection pressure; 17, 18 = negative controls *Phaeoisariopsis griseola* and *A. implicatum* strain 6780 201V, respectively; 19 = positive control pSK1019.

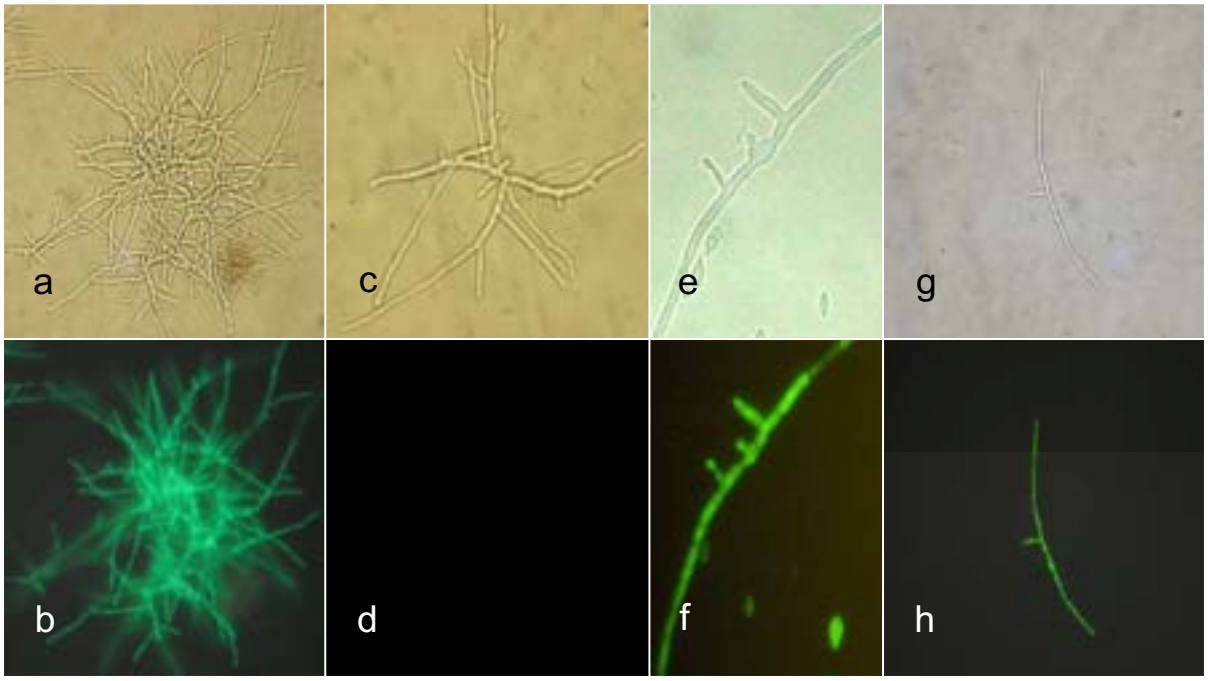

Figure 2.22.4. Structures of *Acremonium implicatum* strain 6780 201v transformed with green fluorescent protein gene (*egfp)* and observed microscopically with UV light. Photos a, c, e and g under normal light; b = fluorescence emission from transformed mycelia under Leica D filter (355 and 425 nm); c and d = control untransformed *A. implicatum* strain 6780 201v without and with UV light, respectively; f and h = transformed structures emitting green fluorescence under UV light with H3 filter (420 and 490 nm).

Microscopic examinations of selected transformants demonstrated strong expression of *egfp* as evidenced by the intense fluorescence emission. All parts of the fungal structure including conidia, mycelia, germinating conidia showed emission. These results demonstrate that the fungal promoter glyceraldehyde-3-phosphate dehydrogenase (GPD) isolated from *Cochliobolus heterostrophus* functions well for expression of genes in the endophytic fungus *A. implicatum* (Figures 2.22.4 and 2.22.5).

The mitotic stability of the transferred DNA was examined by growing 10 transformants in liquid and agar media for 6 generations without any selection pressure. In all cases, resistance to hygromycin B was maintained indicating that the transferred DNA was stable. They all retained emission of fluorescence as well. The meiotic stability could not be determined because the fungus cannot be crossed.

Preliminary data showed that *Brachiaria* tissues taken from plants inoculated with GFP-transformed *A. implicatum* expressed fluorescence emission (Figure 2.22.6). Figure 2.22.5 shows the gfp-expressing transgenic *A. implicatum* used to inoculate *Brachiaria* plants. This will allow us to study the endophyte-*Brachiaria* interaction, endophyte distribution within the plant tissue, and stability in the seed. This will in turn allow us to examine the potential use of this endophyte as a gene delivery and expression system in plants.

Although various transformation systems have been developed and reported for many fungi, successful application of the technology is still not routine in many species. Furthermore, developing an efficient transformation system for a previously untransformed fungus can be a technical obstacle. This work

describes the transformation and expression of the GFP-encoding gene in an isolate of *A. implicatum*, an endophyte in species of *Brachiaria*. We have demonstrated that both the mycelia and conidia of *A. implicatum* can efficiently be transformed using *A. tumefaciens*. To the best of our knowledge, this is the first report on transformation of this endophytic fungus.

The stable integration and expression of the introduced gene into the genome of the recipient fungus indicate that the endophyte may be an excellent tool for delivering and expressing genes of agronomic importance such as disease and insect resistance to host plants. For this to be successful, the practical implication of high seed transmission of *A. implicatum* in *Brachiaria* is significant: once associated with the plant, the fungus can perpetuate itself through seed, especially in apomictic genotypes of *Brachiaria*, for as long as seed storage conditions do not diminish the survival of the fungus (Dongyi and Kelemu, 2004, Plant Disease 88:1252-1254) Several *Brachiaria* hybrids obtained from CIAT's forage breeding program were shown to harbor *A. implicatum*. In addition, we want to exploit the qualities of GFP as a reporter and study the interactions between *A. implicatum* and its host *Brachiaria*.

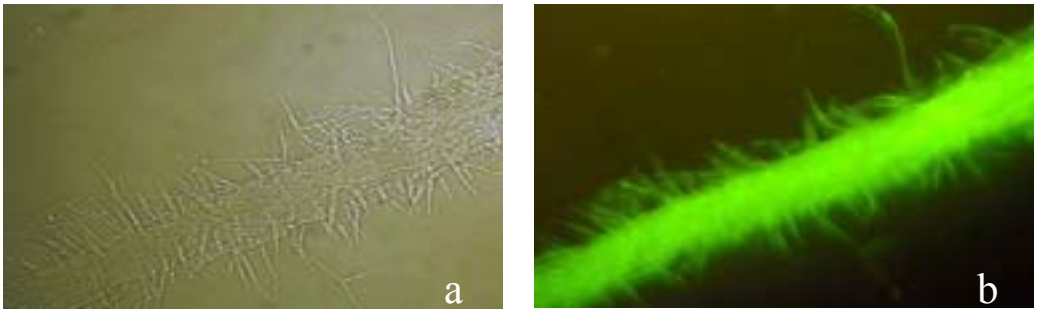

Figure 2.22.5 Mycelium of *Acremonium implicatum* transformed with enhanced green fluorescent protein (*egfp)* encoding gene: a) mycelium observed microscopically (40x) under normal lighting, b) the same mycelium observed under UV lighting, and demonstrating fluorescence emission.

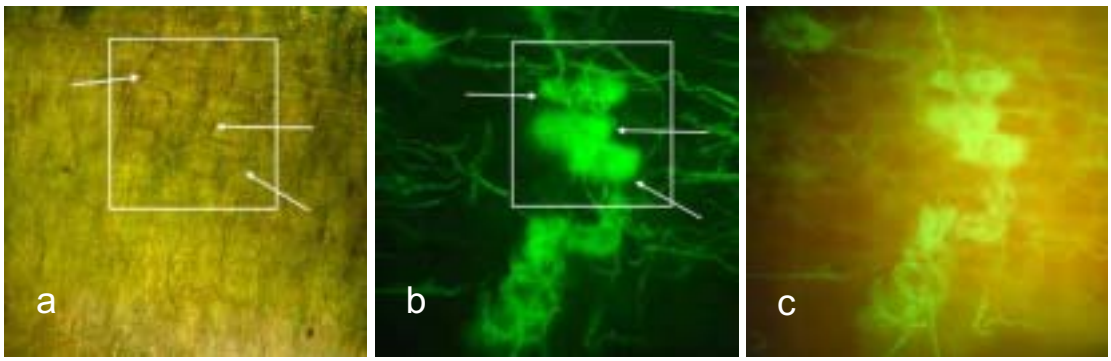

Figure 2.22.6. *Brachiaria* tissues from plants inoculated with *Acremonium implicatum* strain 6780 201v transformed with green fluorescent protein gene (*egfp*) [transformed strain shown in Figure 4). a) under normal lighting, b) fluorescence emission under UV light with Leica D filter, c) fluorescence emission under UV light with Leica H3 filter

Activity 2.23. An antifungal protein isolated from seeds of the tropical forage legume *Clitoria ternatea* controls diseases under field and greenhouse conditions

Contributors: G. Segura, S. Kelemu, and G. Mahuku

Rationale

Seeds use strategies such as production of antimicrobial and/or insecticidal proteins to germinate and survive in soils that are densely inhabited by a wide range of microfauna and microflora. Antimicrobial proteins and peptides have been isolated from seeds of maize (*Zea mays L.*), radish (*Raphanus sativus L.*) and various other plants. They are believed to play a role in plant defense because of their strong antimicrobial activity. This belief is supported by their ability to confer resistance (to pathogens) to transgenic plants containing genes that encode them.

In a previous study, we examined seeds from several tropical forage legumes, for antifungal properties. Of those examined, we isolated, purified, and characterized a protein, designated 'finotin', from seeds of Clitoria ternatea (L.) that exhibited, in vitro, strong antifungal activity on the test fungus Rhizoctonia solani Kühn (Kelemu *et al*., 2004, Plant Physiology and Biochemistry 42: 867-873). This protein has antifungal, antibacterial and insecticidal properties.

In this study, we examined the potential use of finotin as a biopesticide for disease control under field and greenhouse conditions.

Materials and Methods

Treatment of P. griseola conidia with the protein finotin: Twenty-σl of a conidial suspension (10^{-4}) was placed on a slide and subsequently covered with a thin layer of potato dextrose agar medium. A 200-σl crude antifungal protein preparation (the same concentration that was used to spray onto bean plants) was applied on the agar. Protein preparation protocols were as described previously (Kelemu *et al*., 2004, Plant Physiology and Biochemistry 42: 867-873). Control slides had only water. These were placed in Petri dishes containing wet filter paper and incubated at room temperature. Pictures of conidia were taken under the microscope at 0, 32 and 96 hours to observe the development of individual conidia.

Plant inoculation and extract applications: A highly virulent isolate of the pathogen *Phaeoisariopsis griseola*, causal agent of angular leaf spot, was grown on V8 agar at 24^{o}C for 12 days. Conidia were collected and suspended in sterile distilled water at a concentration of 2 x 10^4 conidia per mL. This inoculum was used on *Phaseolus vulgaris* variety Sprite (a susceptible one) bean plants.

*Greenhouse testing**:*** Seventeen-day old bean plants (15 plants per treatment) were sprayed with either the fungicide benlate (500 εg/ml), crude antifungal protein preparation, or sterile water. Two hours later all the plants were inoculated with *P. griseola* conidia (2 x 10^4 conidia per mL). The inoculated plants were placed in a humidity chamber for 4 days, then transferred to the greenhouse for symptom development. Treatments with crude antifungal protein, benlate or sterile water continued every 2 days. Disease evaluations were conducted 10 days after inoculation.

*Field testing**:*** Thirty days old seedlings of tomato variety Manalucie were transplanted to the field in a randomized design with 3 replications (8 plants per treatment in each replication). Treatments were; 1) control treatment with water alone, 2) spray application (till plants were completely wet) of crude protein preparation once a week, and 3) spray application of crude protein twice a week. Various diseases developed under natural infections.

Results and Discussion

Effect of antifungal protein Finotin on bean angular leaf spot: The crude protein extract from seeds of *C. ternatea* CIAT 20692 showed antifungal activity *in vitro* on the pathogen *P. griseola* (data not shown). Conidia treated with the crude protein failed to germinate 32 or 96 hours after treatment (Figure 2.23.1). Plants treated with the crude antifungal protein preparation consistently developed fewer angular leaf spot disease lesions than the control plants that were treated with sterile distilled water (Figure 2.23.2). Had a purified protein been used to control the disease on bean plants, the level of disease control would perhaps have been even higher.

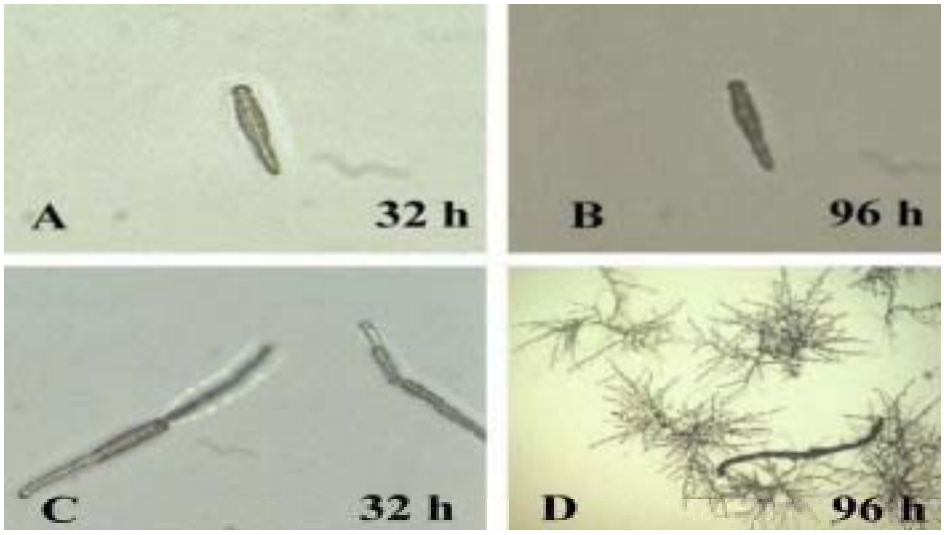

Figure 2.23.1. Treatment of *Phaeoisariopsis griseola* conidia with the antifungal protein finotin. Conidia failed to germinate in the presence of the antifungal protein finotin, 32 and 96 hours (A and B) after treatment, whereas those treated with sterile water germinated (C and D). [Annual Report 2004].

Effect of antifungal protein finotin on tomato diseases: Tomatoes are generally susceptible to a number of diseases under natural conditions. The purpose of these experiments is to develop a simple disease control strategy for small producers using this antifungal protein. Plants sprayed with the crude protein preparation once or twice a week developed better, had fewer disease symptoms, had more plant biomass, and produced more tomatoes than control plants (Figures 2.23.3, 2.23.4).

The protein finotin, is shown to be inhibitory to the growth of a range of important plant pathogenic fungi and at least one important bacterium pathogenic to common bean, as well as two important species of bruchids, *Z. subfasciatus* and *A. obtectus* (Kelemu *et al*., 2004, Plant Physiology and Biochemistry 42: 867-873). These findings raise the possibility that finotin may contribute to the high level of disease and insect resistance observed in *C. ternatea* in the field.

Finotin is released from seeds when the seed coat is mechanically damaged creating a zone of fungal growth inhibition *in vitro*. The antifungal activity of finotin is not affected by high temperatures, which made attractive for the direct use of this protein in disease management under field and greenhouse

conditions. The results presented here demonstrate that a disease control strategy can be developed for small producers using this antifungal protein.

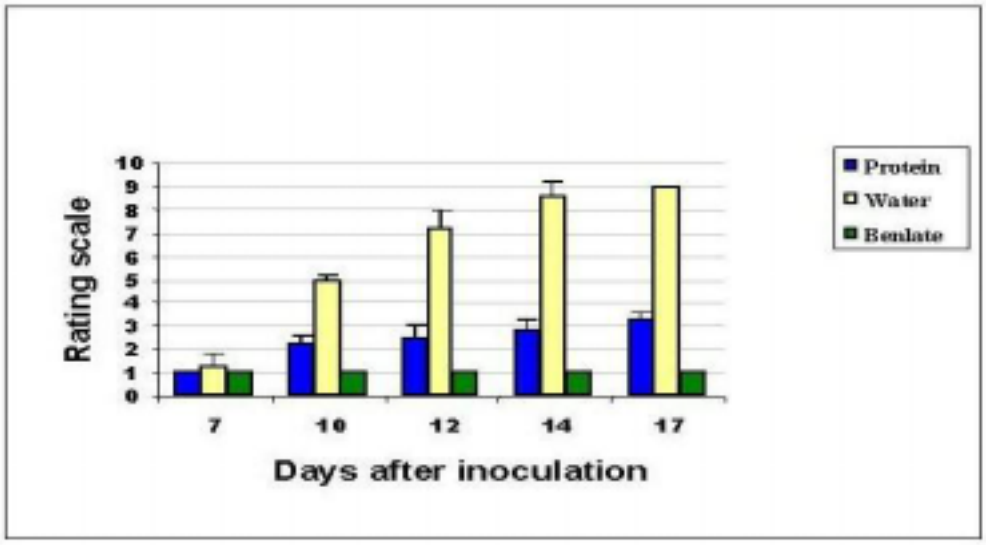

Figure 2.23.2. Angular leaf spot disease development in artificially inoculated bean plants following treatment with crude antifungal protein preparations isolated from *C. ternatea* CIAT 20692, the fungicide benlate, or water control (from AR-2004).

Figure 2.23.3. Tomato plants sprayed with crude antifungal protein preparations twice (3), or once (2) a week, and control (water) [1].

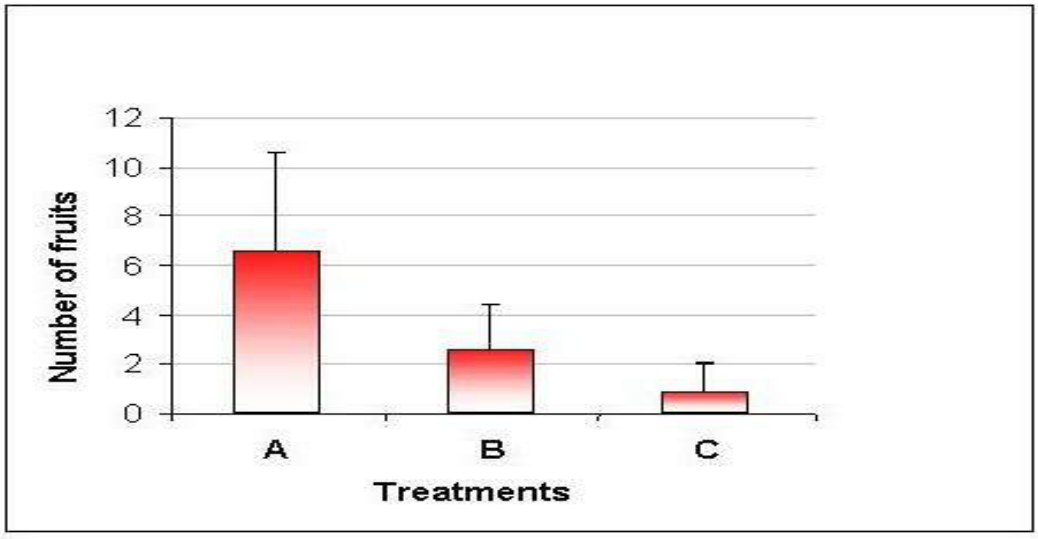

Figure 2.23.4. Average tomato fruit yield per tomato plant in plants treated with crude protein preparations twice a week (A), once a week (B) and water only (C).

Activity 2.24 . Isolating the gene encoding a biocidal protein named Finotin

Contributors: Martin Rodriguez and S. Kelemu

Rationale

Diseases and pests are major biological production constraints in a wide-range of crops. Plants, when attacked by harmful agents, can trigger an array of defense mechanisms. Pathogens and pests, in turn, have an array of matching mechanisms and evolve to overcome and compromise plant defense systems. One type of plant defense mechanism is the synthesis of proteins/peptides or low-molecular weight compounds following mechanical wounding or attack by biological agents. Biocidal or antimicrobial proteins are widely distributed in nature and are synthesized by various organisms. A number of plant-derived proteins that have antimicrobial or insecticidal properties have been isolated and characterized from various plants. One such example is the isolation, purification and characterization of a highly basic small protein, designated 'finotin', from seeds of *Clitoria ternatea* (Kelemu *et al*., 2004, Plant Physiology and Biochemistry 42: 867-873). This protein has broad and potent antifungal, antibacterial and insecticidal properties, indicating that it may contribute to the high level of disease and insect resistance observed in *C. ternatea* in the field. We have subsequently demonstrated that plants sprayed with the crude protein preparation consistently developed fewer lesions of various diseases than the control plants both in greenhouse and field experiments (Kelemu *et al*., 2005, Phytopathology 95:S52).

In light of these findings, it is important to isolate the gene encoding finotin for application of non-host resistance in various crops to combat diseases and pests of economic importance. We report here the progress we have made towards polymerase chain reaction-based cloning of a cDNA corresponding some amino acid sequences of the protein.

Materials and Methods

*Plant material***:** Fully-developed but not dried seeds of *Clitoria ternatea* CIAT accession #20692 were collected directly from the pods and used in this study.

RNA Extraction: Various extraction methods described by several authors were evaluated. Of those evaluated, the methods described by Azevedo *et al* (2003, Plant Mol Biol Reporter 21: 333-338), and Chang *et al* (1993, Plant Mol Biol Reporter 11: 113-116) resulted in good quality RNA comparable to that obtained with an RNA isolation kit from Promega. mRNA was isolated from this total RNA using Oligotex™ Direct mRNA kit (QIAGEN) according to the manufacturer's instructions.

*Synthesis of cDNA***:** Although there are various methods for doing so, complementary DNA (cDNA) is often synthesized from mature (i.e. fully spliced) mRNA using reverse transcriptase enzyme, which operates on a single strand of mRNA and generating its complementary DNA based on the pairing of RNA base pairs (A, U, G, C) to their DNA complements (T, A, C, G). In this study, the synthesis was conducted using 200 ng mRNA, 12-σM of BD SMART II**™** A oligonucleotide and 12-σM of *Primer* 5'-RACE CDS in a 10-σl volume. This was incubated at 70°C for 2 min, then placed on ice. Two-σl of 5x buffer, 1-σl of DTT (20 mM), 1-σl of 10 mM dNTP and 1-σl of BD PowerScript Reverse Transcriptase were subsequently added to the mixture and incubated at 42°C for 2 h (the reverse transcriptase scans the mature mRNA and synthesizes a sequence of DNA that complements the mRNA template). Fifty-σl of Buffer Tricina-EDTA was added and further incubated at 70 °C for 7 minutes to deactivate the reaction. For synthesis of cDNA for the 3´ end, 500-ng mRNA and 1-σl (100-ng/σl) of primer oligo-$(dT)_{25}$ were mixed and incubated at 70°C for 8 min followed by cooling the mixture on ice. Subsequently, 10-σl of 5X buffer, 1-σl of 25mM dNTP, 2-σl of 100 mM DTT and 1-σl of SuperScript™ III RT (200 U/σl) were added to the mixture and incubated for 1 h at 50°C. At the end the mixture was deactivated by heat treatment at 70°C for 10 min. Information on oligonucleotides used in this study is given in Table 2.24.1.

Table 2.24.1. Sequences of primers and adaptors used in this study.

Name	**Sequence**
Oligo dT	5'-$(dT)_{20}$-VN*-3'
5' RACE CDS-primer A	5'-$(T)_{25}$-VN*-3'
BD SMART II™ A Oligonucleotide	5'AAGCAGTGGTATCAACG- CAGAGTACGCGGG 3'
primer UPM (Universal Primer A Mix)	5'CTAATACGACTCACTATAGGGCAAGCAGTGGTA TCAACG CAGAGT3'
primers NUP (Nested Universal Primer A)	5'AAGCAGTGGTATCAACGCAGAGT3'
MERF 1	5'-TGYGARCGNGCNTCNCTNACNTGG-3'*
MERF 2	5'-GARMGNGCNWSNYTNACNTGGACN-3'*
MERF3	5'-ACNGGNAAYTGYGGNAAYACNGGNCA-3'*
MERF4	5'-AAYYTNTGYGARMGNGCNWSNYT-3' *
MERF5	5'-ACNTGGACNGGNAAYTGY-3' *
FINOR 5	5'-CARTCRAARTARCARAARCAYTT-3'*
FINOR 6	5'-RTTNCCNCKYTTRTGRCANGCNCC-3'*
** N=A, C, G o T; V=A, C o G H=A, C o T D=A, G o T R=A o G Y=C o T M=A o C*	

*Polymerase chain reaction of cDNA***:** We contracted Cornell University's biotechnology unit to sequence finotin. However, the protein (finotin) sequence data obtained from Cornell Biotech was not satisfactory,

and as a result we used sequences of an antifungal protein (from Clitoria ternatea) reported by Osborn *et al* (1995, FEBS Lett. 368: 257-262) to generate degenerate primers. A 25-σl PCR mixture contained 1.5-mM of $MgCl_2$, 200-ε Mof dNTPs, 0.5-ε M of each olgonucleotide (UPM and FINOR5), 200-ng cDNA, 1 unit of Taq DNA polymerase, and 1x PCR buffer. Amplifications were programmed with 35 cycles of a 30 second (3 min for the first cycle) denaturation step at 94 °C, annealing for 45 seconds at 50 °C, and prime extension for 45 seconds at 72 °C.

Cloning and sequencing: The amplified product was excised and eluted from the agarose gel using a BIO-RAD DNA purification kit. This was cloned in pGEM-T Easy vector (Promega, USA), and sequenced using ABI Prism 377-96 DNA Sequencer. The sequence data were aligned using Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI).

```
1          acgcggggatagtagataagagtaaataaggtagctagcttagtacctggtttaaagtta
61         aggagagtATGGCAAAGTGTAACACAATGGTAATAGCATTAGCAGCAGTAGTAGTAGTGT
1                    M  A  K  C  N  T  M  V  I  A  L  A  A  V  V  V  V
121        TGCTGATTGATGGTGGAGAAAGTTTTGCAATATGTAACGTAGATTCAAGTCAGTTAAGCT
18         L  L  I  D  G  G  E  S  F  A  I  C  N  V  D  S  S  Q  L  S
181        TGTGTCGTGCAGCAGTTAGTGGTGGTAATCCGCCACCACCAGATGAAAAGTGTTGTGCTG
38         L  C  R  A  A  V  S  G  G  N  P  P  P  P  D  E  K  C  C  A
241        TCATTCGTCAGGTCAATCTGCCCTGCCTCTGCCAATACAGGGGATTCCTACTTCGGTTTG
58         V  I  R  Q  V  N  L  P  C  L  C  Q  Y  R  G  F  L  L  R  F
301        GAATCAATCCCAAAAATGCTTTTGCTACTTCGACTG
78         G  I  N  P  K  N  A  F  A  T  S  T
```

Figure 2.24.1. Sequence analysis of a cDNA clone synthesized from mRNA of seeds of *Clitoria ternatea.* The figure shows nucleotide sequences and the corresponding deduced amino acid sequences The sequence highlighted in green represents a possible TATA box sequence where as the yellow highlighted region represents a sequence commonly present in various LTP genes. The cysteine amino acid sequences are circled. The sequence of primer FINOR5 (anti-sense) is underlined.

Results and Discussion

Amplified DNA fragments (using cDNA as a template) ranging from 120 to 650 bp were isolated and cloned. Ten combinations of primers, 7 sense and anti-sense orientations and a universal primer, were used on 4 different preparations of cDNAs. A total of 37 clones were generated and of these, 17 have been sequenced so far. The sequence of one such clone is shown in Figure 2.24.1. The sequence data demonstrated homology to genes encoding nonspecific lipid transfer proteins (nsLTPs) from plants. These findings may be significant because nsLTPs have been reported to play a role in plant defense systems. A number of peptides (small proteins with sizes ranging 2-10 kDa) including nsLTPs have been reported to be involved in plant defense mechanisms. It is generally believed that seed proteins with antimicrobial activity may play a role in the protection of seeds against harmful microbes. Nonspecific lipid transfer proteins are basic, 9-kDa proteins with conserved cysteines and present in high amounts in plants. One promising clone with homology to nsLTPs is tentatively designated CtLTP. The clone is not complete, but based on sequences from nsLTP of other plants, only a small portion from the 3' end is missing (Figure 2.24.2).

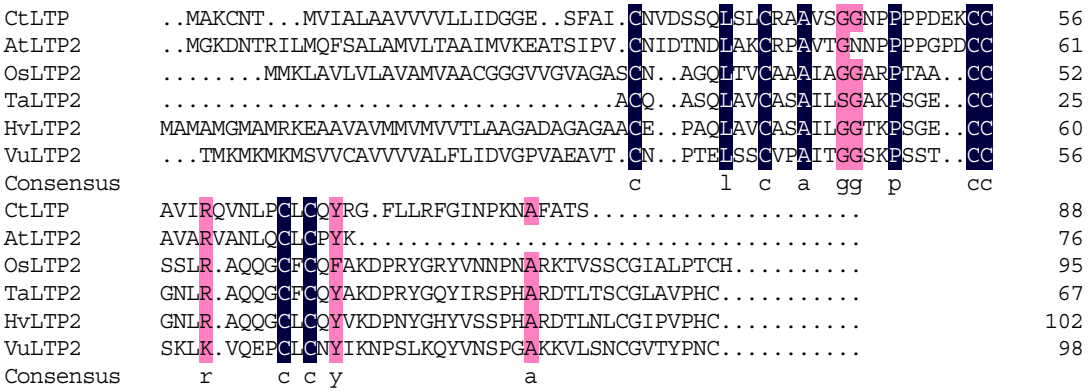

Figure 2.24.2. Amino acid sequence comparisons among various plant nsLTPs that are associated with plant defense systems. CtLTP: sequences deduced from this study; AtLTP: nsLTP of *Arabidopsis thaliana*; OsLTP: nsLTP of *Oryza sativa*; TaLTP: nsLTP of *Triticum aestivum*; HvLTP2: nsLTP of *Hordeum vulgare*; VuLTP2: nsLTP of *Vigna unguiculata*.

The biological function of nsTLPs is still well understood. However, a number of studies have demostrated growth inhibition of a range of pathogens by nsTLPs. For example Cammue *et al* (1995, Plant Physiol. 109:445-455) isolated a protein from seeds of onions (*Allium cepa* L.) that had sequence homology to nsLTPs and that was a potent groth inhibitory effect against 14 different pathogens.

Our preliminary data presented in this report indicate that the cDNA clone that we generated may be classified as a member of the nsLTP protein family based on the deduced amino acid sequence. Work is in progress to generate a full length cDNA clone, to complete the sequences of the remaining 20 clones, and to determine the antimicrobial activity of a successful complete clone.

Activity 2.25. Inducing symptoms of bacterial wilt of plantain with a strain of *Ralstonia solanacearum* isolated from tomato

Contributors: Elizabeth Álvarez, Germán Llano, Juan Pablo Castillo, and John Loke

Highlight:

- First occurrence of an emerging tomato strain of *R. solanacearum* race 1, a new pathotype that genetically clusters with plantain strains (race 2). Greenhouse inoculation showed that the tomato strain was pathogenic to plantain, hence, bringing into question the use of plantain–tomato rotations.

Rationale

The *R. solanacearum* species complex, which includes the banana blood disease bacterium (BDB) and *Pseudomonas syzygii*, is a highly diverse group of organisms. Traditional methods of characterizing this organism, including biovar typing and race assessment, have been very useful in defining diversity within *R. solanacearum*. However, recent genetic evidence has indicated that these phenotype-based schemes are not sufficient to cover the entire diversity of strains represented by the species *R. solanacearum*. In our study, the pathogenicity of *R.* solanacearum obtained from tomato was determined in plantain seedlings, confirming Fegan and Prior's hypothesis (Fegan and Prior, 2002, 3rd International Bacterial Wilt Symposium, South Africa).

Materials and Methods

Samples of leaves and stem bases were taken from an infected plant growing in a tomato crop with symptoms of wilt. The crop was located on a farm in the Municipality of Montenegro (Quindío) and was being cultivated beside a plantain crop that had foci of bacterial wilt. The samples were processed in the laboratory. They were first washed for 20 min under running water, disinfected with 1% sodium hypochlorite for 1 min and then in 50% alcohol for another minute. They were then rinsed twice with sterilized distilled water, and finally macerated in sterilized distilled water. The mash was cultured onto SMSA medium and incubated at 28 ∀C for 4 days.

The mucoid and reddish colonies grew in the medium, and were purified by droplet transfer to SMSA. After 3 days, they were transferred to nutrient agar (NA) medium, where the bacterium grew for 36 h. A bacterial suspension was then prepared with the bacterium grown on NA by scraping the dishes in sterilized distilled water and passing through a spectrophotometer at an absorbance of 0.1 at 600 nm wavelength (1×10^8 cfu/mL). This suspension was injected at 0.5 mL/plant into 4 seedlings of plantain variety Africa. The control used was strain CIAT 1008, isolated from plantain. The inoculated plants were incubated for 5 days with continuous wetting at 22 ∀C at night and 30 ∀C during the day, and 95% relative humidity, after which they were moistened daily for 1 h.

Once the symptoms were reproduced in the plantain, the bacterium was re-isolated. The pathogenic strains were again inoculated into plantain and into 15-day-old tomato seedlings by injection of a bacterial suspension.

In another trial, the soil was inoculated with a bacterial suspension of strain CIAT 1008 at 0.1 absorbance at 600 nm wavelength in a 25-mL volume that was added to 1-kg flasks carrying 30-day-old tomato plants. In some flasks, perpendicular cuts were made into the soil, 5 cm from the stem thus causing root wounds. This treatment was compared with unwounded plants. Plants were also inoculated by injection of 0.5 mL of the bacterial suspension. The plants were incubated as described for plantain.

Results and Discussion

Symptoms began appearing in plantain plants in the second week after inoculation. The plants showed wilting of leaves and later death in a manner very similar to that of the control (Figure 2.25.1). Before the plants totally wilted, the bacterium was re-isolated and cultured onto SMSA, where the typical morphology of *R. solanacearum* was observed. The inoculated tomato plants showed wilting within 6 days and died 3 to 5 days later.

Figure 2.25.1. A) Bacterial wilt in tomato cultivated in the plantain-growing area of Montenegro (Quindío, Colombia). B) Symptoms of bacterial wilt are reproduced in plantain seedlings inoculated with a strain of *Ralstonia solanacearum* isolated from the tomato plants observed in A.

Plants that received the soil inoculation treatment causing root wounds showed typical symptoms of bacterial wilt 6 days after inoculation. The plants that were not wounded showed symptoms 4 days later (Figure 2.25.2).

Figure 2.25.2. Bacterial wilt in tomato caused by isolate CIAT 1008 of *Ralstonia solanacearum* obtained from plantain, 10 days after inoculation. A) Plant with wounds to the roots. B) Plant without wounds to the roots

The results contradicted reports that suggested that race 2 is specific to the Musaceae and does not attack tomato. On the contrary, they confirmed Fegan and Prior's findings where they had identified several phylotypes of *R. solanacearum* attacking both plants. The strains we isolated from both plantain and tomato classified as phylotype II.

Activity 2.26. Evaluating ecological practices of soil management in foci affected by bacterial wilt (*Ralstonia solanacearum*) in two plantain crops in the Department of Quindío

Contributors: E. Álvarez, G. Llano, M. Vargas, O. Zuluaga, L. A. Meza Becerra, V. H. Treviño Henao, J. L., and J. F. Mejía

Rationale

Farmers consider current recommendations for managing moko as inefficient and demand that formalin, used to disinfect soil, be replaced with a nontoxic alternative. In earlier work, our greenhouse experiments have shown that French marigold (Tagetes patula) reduces bacterial populations in the soil by 85%. To evaluate the effect of incorporating into the soil several different nontoxic alternatives to inhibit *Ralstonia solanacearum*, field experiments were conducted in the Quindío region of Colombia.

Materials and Methods

Field: Three foci affected by bacterial wilt were chosen in two plantain crops on "La Guaira" and "Cataluña" Farms (Montenegro, Quindío). To evaluate their effects on the bacterium *R. solanacearum*, causal agent of bacterial wilt, the following treatments were applied:

- Phosphoric rock (29% P_2O_5), lixiviate, and entire *T. patula* plants were incorporated at rates of 25 kg, 24 L, and 4 kg, respectively, at each affected site

- Check with 50% formalin

- Check with no treatment

The treatments were distributed in a randomized complete block design, with 3 replications in "La Guaira" and 6 in "Cataluña". The experimental unit corresponded to an affected site (i.e., one plant with daughter and granddaughter).

Before applying treatments, samples were collected from soil at 20–25 cm deep and from affected plants in 25-cm-long pieces that encompassed both above and below ground parts, as well as tissues. The samples were used to detect the bacterium.

Once sick plants in the foci were identified, these plants were uprooted and chopped into pieces of about 30 to 40 cm long, stacking them on the affected site. Above the pieces the phosphoric rock was applied first, then the lixiviate, and finally the *T. patula* plants, cut into pieces on application to favor release of the plants' thiophenes, which are probably responsible for inhibiting the bacterium (Arenas *et al*., 2005, Fitopatol Colomb 28:76–80.). The mixture was covered with soil and with black polyethylene to prevent weeds growing.

To apply formalin to the affected site, the soil was perforated at five points with a ¼-inch-diameter rod to as deep as 60 cm. Two liters of 50% formalin were applied, filling up every orifice. The treated site was then covered with black polyethylene.

The check consisted of plant material that was chopped up and covered with black polyethylene, receiving no treatment.

Once the treatments were applied, a ditch was dug around each site to prevent contamination among sites.

Every 30 days, samples were taken of the soil at 20 and 50 cm deep in the same site where the affected plant was found. Three months after the trial was established, the treatments were re-applied.

Isolation: From each soil sample, 3.3 g were taken and 30 mL of TE buffer (10 mM blend of TRIZMA® base and TRIZMA® HCl, and 1 mM of EDTA) at pH 7.6 were added and homogenized by vortexing. This suspension became the base solution.

From this base solution, dilutions at 10^{-1} to 10^{-3} strengths of TE buffer were prepared. Of each dilution, 100 εL were placed in petri dishes containing SMSA (1 liter of medium contained 10 g Bacto™ Peptone, 5 mL glycerol, 1 g casamino acids, 18 g Bacto™ Agar, 26 mg bacitracin, 100 mg polymyxin-ϸ sulfate, 5 mg chloramphenicol, 0.5 mg penicillin, 5 mg crystal violet, and 50 mg 2,3,5 chlorotriphenyltetrazole). The samples cultured onto this medium were incubated at 28 ∀C for 7 days.

After 7 days, colonies that looked like *R. solanacearum* (reddish color, mucoid, and amorphous) were transferred to SMSA and incubated for 4 days. Colonies confirmed as possibly positive were transferred to nutrient agar (20 g/L), incubated at 28 ∀C for 24 h, and inoculated onto plants of plantain variety Africa that had derived from meristem culture.

Inoculation: For inoculation, a bacterial suspension of 0.1 absorbance at 600 nm was prepared and 0.5 mL injected into each plant. The inoculated plants were incubated for 10 days in a humid chamber at 29

∀C during the day and 24 ∀C at night and relative humidity between 80% and 91%. From day 10 to day 30, records were made of those plants that produced typical symptoms of bacterial wilt. Detection by PCR was also carried out with specific primers, as described in Activity 2.

Indicator plants: Three methodologies were carried out for evaluations with indicator plants:

1. In one focus in "La Guaira" Farm, with two replications, 55 plantain plants were planted in an area of 4 × 4 m to act as indicators of the presence of *R. solanacearum* in the soil.

2. Because the bacterium was difficult to isolate from the soil, apparently because of its small population, 4 plantain plants were planted in each experimental unit 4 months after the trials were established.

3. Fegan and Prior (2005, *In*: Allen *et al*. (eds), Bacterial wilt disease and the *R. solanacearum* species complex, APS press, pp 449–461) reported phylotype II of *R. solanacearum* as being infectious in tomato and plantain. Although no other report is known in Colombia, a tomato growing in Montenegro (Quindío) was found to have symptoms of bacterial wilt, infected by the same phylotype of *R. solanacearum* pathogenic in plantain. Based on this report, tomato plants were also planted as indicators, on each site.

Results and Discussion

In direct form, the soil samples were detected as having very low levels of the bacterium, even in the check with no treatment. Hence, 1 mL of each of the base solution and 10^{-1} to 10^{-3} dilutions were mixed with 1 mL of liquid broth medium to enrich the bacterial population before culturing onto SMSA medium. Of the samples collected before applying the treatments, the bacterium was detected in two sites in each of "La Guaira" and "Cataluña" Farms.

At 1 and 5 months of applied treatments, the bacterium was detected in a check established on the "Cataluña" Farm. The presence of the bacterium in these samples was verified by inoculating plantain and amplifying through PCR.

At 4 months after establishing the trial in "La Guaira", one of the indicator plants (planted in 4 m square) manifested symptoms of bacterial wilt. This plant was located on the margin of a ditch that separated a check treatment with no applications.

After 8 months of applying the treatments in "Cataluña", four indicator plantain plants, planted on the site, were recorded as infected. These plants corresponded to two treatments with formalin and two controls, indicating a possible re-inoculation of the bacterium. In "La Guaira", no new infected plants were detected.

Output 3: Strengthened capacity of NARS to design and execute IPM R&D, to apply molecular tools for pathogen and pest detection, diagnosis, diversity studies and to device novel disease and pest management strategies.

Activity 3.1. Developing integrated pest management components

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Highlight:

- Continued and expanded diffusion of technology activities within the DFID-funded project on Sustainable Management of Whiteflies

Rationale

Whiteflies have become the target of excessive pesticide use by snap bean and dry bean farmers in the Andean zone. A management system for whiteflies that contribute to reduce pesticide use has been developed and tested with farmers in Colombia and Ecuador (see 2002-2004 PE-1 Annual Reports). In 2005 we continued and expanded diffusion of technology activities at both sites in Colombia and Ecuador. Work was initiated at a third site in Bolivia.

Materials and Methods

Following the renewal of the DFID project, diffusion activities were planned and contacts with collaborating partners were made. Preparation of technical and extension bulletins was initiated. New collaborators in Bolivia were trained. A large-scale demonstration trial was conducted in the Tenerife area of Colombia.

Results and Discussion

The large-scale demonstration plot allowed us to validate with success, in a new site, the technology that had been developed in previous years. As in previous trials, and as compared with farmers' practices, alternative management strategies based on judicious timing of applications and use of action thresholds resulted in yields that did not differ from those obtained by farmers with their traditional management approaches (Table 3.1.1). We were able to demonstrate that with a combination of cultural practices, the use of systemic insecticides as seed dressing and proper timing of foliar applications farmers can obtain higher benefit/cost ratios with a 60-70% reduction in the amount of applications made per cropping cycle.

Other activities were:

1. Training of Vladimir Lino (Proinpa), a new collaborating partner in Bolivia
2. Reinitiating Farmers Schools activities in the Chota region of Ecuador
3. Writing, editing and printing of a technical bulletin on management of whiteflies (Figure 3.1.1) and extension bulletins for Colombia, Ecuador, and Bolivia (Figure 3.1.2).

Table 3.1.1. Yields (Kg/ha) and economic returns obtained with two approaches for control of the greenhouse whitefly *Trialeurodes vaporariorum* in Tenerife, one of the reference sites in Colombia. Unreplicated demonstrative trial. No statistical analysis performed.

Treatment	No. of insecticide applications	Yield (Kg/ha)	Costs ($/ha)		Benefits ($/ha)		Benefit/cost ratio
			Variable	Total	Total	Net	
CIAT's proposal[a]	3	9327	275	1655	4145	2490	2.50
Farmers practices[b]	7	7998	408	1788	3554	1767	1.99

[a] Seed treatment with imidacloprid followed by two foliar applications of conventional insecticides at pre-established action thresholds; [b] 6-7 foliar applications of conventional insecticides.

Figure 3.1.1. Title page of the technical bulletin produced in 2005. This publication will be extensively used in diffusion of technology activities during 2006 and beyond.

Figure 3.1.2. Title pages of extension bulletins produced in 2005. These publications will be extensively used in Colombia (A), Ecuador (B), and Bolivia (C).

Activity 3.2. Diagnosing plant diseases and technical assistance

Contributors: E. Álvarez, J. F. Mejía, G. Llano, J. P. Castillo, E. Gómez, and J. Loke

Rationale

Plant disease diagnoses are carried out by the Cassava Pathology Program as a service to help Colombian institutions and individuals diagnose plant disease problems in their crops. Diagnoses are based on visual observations of symptoms expressed by infected plants and additional laboratory analysis.

Bacteriological and fungal diagnoses were performed on different samples obtained from Colombian farmers and institutions (Table 3.2.1).

Table 3.2.1. Bacteria and fungi isolated from different crops and identified at the CIAT Cassava Pathology Laboratory, Palmira, Colombia.

Location	Host plant	Disease	Detection method	Microorganism identified
Rozo, Valle del Cauca	*Heliconia*	Bacterial wilt	Isolation on selective medium, pathogenicity, and DNA sequence analysis	*Ralstonia solanacearum*
Montenegro, Quindío	Plantain	Bacterial wilt	Isolation on selective medium, pathogenicity, and DNA sequence analysis	*R. solanacearum*
Popayán, Cauca	Tomato	Bacterial speck	Isolation	*Pseudomonas syringae* pv. *tomato*
Montenegro, Quindío	Squash			*Xanthomonas sp.*
Buga, Valle del Cauca	Cacao	Moniliasis		*Moniliophthora roreri*
Tauramena, Casanare	Cassava	Cassava bacterial blight	Isolation	*X. axonopodis* pv. *manihotis*
Montenegro, Quindío	Banana	Fusarium wilt	Isolation	*Fusarium oxysporum*
Montenegro, Quindío	Plantain	Bacterial wilt	Isolation on selective medium, pathogenicity, and DNA sequence analysis	*R. solanacearum*
Dapa, Valle del Cauca; Fusagasuga	*Thea sinensis*; Tomato		Isolation	*Colletotrichum*

Activity 3.3. Evaluating novel / innovative approaches in scaling up integrated pest and disease management (IPDM) technologies

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Highlights:

- Novel approaches with potential for promoting IPDM technologies were evaluated at target project sites
- Trained modified farmer field school (MFFS) groups were effective in knowledge sharing and dissemination of IPDM technologies
- Cohesive and dynamic farmer research groups (FRGs, over 300 groups with more than 50,000 well trained farmers) have evolved at project sites in Uganda, Kenya, Tanzania and Malawi
- Farmer to farmer knowledge sharing enhanced technology dissemination and adoption (60-85% adoption rate)
- Farmers used traditional (drama, songs, poems) and conventional (seminars, demonstration and learning plots, field days and visits, radio, promotional materials) pathways in technology dissemination
- The process of learning by doing and seeing succeeded in using the psychology of development that helped to build farmers' confidence in the effectiveness of indigenous practices that they blended with improved technologies
- The approach and processes used in the project has helped to improve the skills of individual farmers and groups in the identification and management of production constraints (such as diseases, insect pests, soil fertility and markets), demand and search for information on solutions and new technologies
- The practical sessions used in implementing project activities helped to empower men and women farmers in the management of their own resources

Rationale

The purpose of the bean IPDM promotion activities was to contribute to the reduction of losses caused by bean pests through effective targeting, dissemination and adoption of integrated pest management strategies that are acceptable t smallholder farmers in eastern, central and southern Africa. Useful practical experiences have been gained, successes achieved and lessons learnt during the promotion o technologies at target sites. There was need to document farmer adoption behaviour and social economic effects on adopters' livelihoods. Such information will form the basis for developing guidelines for adapting useful approaches, methods and processes to scale-up the adoption of IPM and allied technologies. The guidelines will be shared with key partners to exchange positive and negative experiences and to train current and new partners in future active research.

Materials and Methods

The adoption studies were planned and conducted at three project sites each in Uganda, Kenya and Tanzania. The sites are Kabale district in south western Uganda, Kisii and Rachuonyo districts in Nyanza province, Kenya and Hai district in northern Tanzania. Random and purposive random sampling methods were used to gather primary and secondary data. Data were analyzed using the SPSS program. Studies in Dedza district, central Malawi have delayed and are planned to be conducted by end of March 2006.

Formal questionnaires were administered during interviews with individual farmers, farmer groups and key informants. A total of 100 farmers (53 men, 47 women) out of over than 500 were interviewed. In Kenya, 61 farmers (25 men, 36 women) out of 4000 farmers were involved in the study. In northern Tanzania, where over 6000 farmers are aware of bean IPM technologies, the study sample interviewees comprised a total of 236 individual bean farmers (114 men, 122 women) in 39 farmer groups (out of 77) from 27 villages (out of 76).

Results and Discussion

Kabale, south western Uganda: The studies in Kabale and Hai districts have been completed and documented. The data and results from Kisii and Rachuonyo districts in Kenya have been analysed and report preparation is in progress. In Kabale, (Uganda) the effectiveness of using a trained modified farmer field school (MFFS) group as an option of enhancing farmer knowledge and disseminating IPDM technologies to several peer groups was assessed. The potential for participatory farmer research groups (PFRG) was assessed in Hai (Tanzania) and a combination of MFFS and PFRC) was assessed in Kisii and Rachuonyo districts in Kenya. Farmer / community behaviour in adopting or rejecting IPDM technologies was monitored and documented. The effectiveness and impact of project approach and processes used in disseminating and promoting IPDM technologies on the social economic situation of participating and non participating farmers was assessed and documented. A draft guideline for use by partners to scale up the dissemination of IPDM technologies in the region has been prepared.

The results show that once farmers and other stakeholders understood the pest problems and participated in the processes for developing management options, they gained knowledge, experience and confidence that enable them to effectively disseminate the information using diverse pathways at different sites. Such pathways included various traditional (drama, songs, poems and word of mouth) and conventional (training seminars, demonstrations and learning plots, field days and visits, radio, displays, promotional materials, village information centres, radio and TV). Farmer to farmer dissemination of information and technologies was the most commonly used method. New farmers adopted technologies from innovative farmers with slight or no modifications.

Results from Kabale show that there have been a shift from growing beans as a basic food requirement where only a surplus could be sold to a production status intended to earn income (Table 3.3.1).

Table 3.3.1. Importance of beans in the farming system.

Reason	**Before project (2000)**		**After project (2005)**	
	Frequency	Percent	Frequency	Percent
Food only	17	34	16	16
Food and sell some	33	66	72	72
Both food and cash	0	0	11	11
Mainly cash	0	0	1	1

The study in Kabale also showed that the participatory group approach and processes have helped even the farmers who could neither read nor write to learn effectively and be able to also train others informally. In addition, project activities have influenced change in gender related traditions where, more men are now involved in bean production. This shift could be result of the crop becoming an important source of household income especially now when it is recovering from serious disease and insect pest problems and the high demand for food and improved seed.

A variety of IPDM innovations have been adopted and were being practiced by the beneficiaries. It was found that the more farmers are aware of a specified technology and its benefits and they recognize that it can address their need, the more they are likely to adopt and practice it. Adoption was also dependent on the cost and ease of implementing it. The most widely and less adopted technologies are shown in Table 3.3.2.

Beneficiaries and collaborators observed that it was much easier to access information and technologies through groups that individuals. The IPDM groups in particular had accrued numerous benefits to all development stakeholders. Adoption of technologies was more related to access than preference. For example, there was a positive correlation between the use of manure and digging of trenches, and the size of land (Table 3.3.3). The table shows that farmers with more land had adopted the application of manure and digging of soil conservation trenches. However, due to the hilly terrain, manure application was most common near the homestead fields.

All IPDM groups are interested in bean seed production. Groups sell the available seed at group and household level although they all showed concerns that they did not have enough seed. Some members sell seed to acquire money for renting additional land. The stronger groups have entered the seed market through links with local NGOs (such as Africare and CARE).

According to beneficiaries, the MFFS approach had extra advantages over the other approaches. Seventy one percent (71%) of the responses indicated that the MFFS was better than the rest of the approaches. Out of these, practical learning impressed fifty eight percent (58%), 10% confessed to have acquired better understanding while 3% liked the good interaction the approach accorded participants and trainers. Other additional advantages from focus group discussions were:

- The practical sessions helped learners to understand better and acquire skills that they are currently applying in other enterprises
- The testing allowed them a chance to adopt varieties that were doing well in their localities
- The approach enabled those who could neither read nor write to learn effectively and can also train others informally

Table 3.3.2. Technologies adopted by IPDM groups and how they have helped the farmers in Kabale district, south western Uganda

a) Technologies adopted (mostly for purposes of managing BRR)

Technology	How the technology has helped	Sustainability plan
Application of compost manure (46%)	Increased production (26%), soil fertility improvement (6%), reduced disease (5%), cheap (4%) tasty bean leaves, controlled BSM	Dig compost pits (18%) Rare livestock (11%) Plant fodder (6%)
Changed varieties (8%)	Increased yield (4%)	Save own seed (5%)
Use of FYM (64%)	Increase yields (37%), increases soil fertility (8%), cheap (6%), controls pests/diseases (4%)	Rare livestock (19%) Dig compost pits (15%) Plant fodder for animals (6%)
Planting in lines (15%)	Use less seed (3%), get high yield (5%) and reduced time in weeding & harvesting	Continue practice (8%) Planning fields (2%)
Digging trenches & stabilizing bunds with agro-forestry trees and grasses (22%)	Stops erosion (3%), controls floods (9%) provide stakes, firewood and animal fodder & improves soil fertility	Construction of drainage channels (16%)

b) Technologies that were learnt but were being adopted on a relatively small scale

Technology	*How the technology has helped*	*Why limited adoption*
Resting land/fallowing	Rejuvenates soil fertility, increases soil and crop production	Inadequate land, available is continually farmed
Seed selection & separating of varieties	Access to quality seed, increased demand in the market and by other farmers	Little seed available
Controlled soil & bush burning	Reduces erosion, adds manure to the soil	Inadequate knowledge
Use of chemical sprays	Control of pests and diseases, increased production	Inadequate income/expensive
Crop rotation	Control soil exhaustion by limiting heavy feeders e.g. Irish potatoes & wheat	Inadequate land/need for all crops
Rouging volunteer plants (few people)	Controls diseases (mostly used in the Irish potato enterprise)	Volunteers are a source of early food during period of scarcity
Potting tree seedlings	Healthy seedlings produced, easy handling	
Use of inorganic fertilizers	Increased production	Expensive/Inadequate income

Table 3.3.3. Relationship between size of land and adoption of selected soil fertility management technologies in Kabale district, south western Uganda.

Innovation	% age adopting	Average land size (ha)*	%age not Adopting	Average land size (ha)*
Use of FYM	60	2.5	18	2.3
Use of compost manure	43	2.7	35	2.3
Digging of trenches	12	2.7	66	2.5

* A hectare was interpreted to be an equivalent of the size of a football pitch. Respondents had difficulty in estimating the sizes of plots they owned, particularly where they needed to add more than 1 plot to make a hectare

Kisii site, Nyanza province in Kenya: Results on adoption of IPDM technologies by farmers revealed that farmers had identified and ranked bean production constraints that included insect pests and diseases. Among the major insect pests in order of importance according to farmers' responses are aphids, cutworms, bean stem maggots (BSM) and bruchids while diseases in order of importance are bean mosaic viruses, blight, leaf rust, root rots, anthracnose and angular leaf spot. Farmers had also used their own traditional technologies in insect pests, diseases and soil fertility management. The studies also showed the farmers tested and adopted several different technologies that include use of improved crop varieties, regular scouting of pests, timely planting, use of botanical plant extracts (such as *Tephrosia*, *Tithonia*, Marigold, Datura, Neem and dry sisal leaves) use of conventional chemical pesticides and fertilizers, timely weeding and
harvesting, proper drying and clean storage and soil nutrient management (such as use of compost and manure).

The studies further indicated that 85% of interviewed farmers had adopted several of the IPDM technologies that they had tested at a rate ranging from 92-95% for three of the technologies (Table 3.3.4). A detailed analysis showed that over 80% of the farmers had adopted more than 7 IPDM technologies in a period of over 3 years while 65.4% (n=52), 71.7% (n=53) and 71.2% (n=52) had adopted the use of improved varieties, regular scouting and timely weeding, respectively for more than 4 years (Table 3.3.5).

Table 3.3.4. Overall farmer adoption of IPDM technologies at Kisii site, Nyanza province in Kenya.

IPDM technology	Percent adoption	Number interviewed
Use of improved crop varieties	94.4	52
Regular scouting for pests	94.6	53
Timely weeding	92.6	52

Table 3.3.5. Adoption rate for three of the most preferred bean IPDM technologies by farmers in Nyanza province, Kenya between 2000 and 2004.

	IPDM technology					
	Tolerant crop varieties		Timely weeding		Use of botanical pesticides	
Year	Count (n=49)	%	Count (n=49)	%	Count (n=43)	%
2000	15	31.9	15	30.6	13	30.2
2003	38	80.9	28	77.6	34	79.1
2004	46	97.9	48	98.0	42	97.7

The data from three locations at Kisii site also indicated that farmers (76.2%, n=42) had adopted improved been varieties (Table 3.3.6) because they are high yielding; tolerant to insect pests, diseases, drought and infertile soils, early maturing, suitable for food and sale at the local market.

Table 3.3.6. Comparison of farmers growing the most preferred pest tolerant bean varieties in Kasipul, Kabondo and Mosocho divisions in Nyanza province, Kenya.

Bean variety	**Kasipul**		**Kabondo**		**Mosocho**	
	Count	**%**	**Count**	**%**	**Count**	**%**
G8047	10	66.7	22	81.5	2	10.5
EXL52	5	33.3	20	74.1	5	26.3
Red Harricot	6	40	1	0.04	10	52.6
ARA4	4	26.7	6	22.2	10	52.6
KK15					7	36.8
Wairimu	13	86.7	9	33.3	3	15.8
EXL55	5	33.3	14	51.9	2	10.5
GLPS Nyayo (Lyamungo 85&90)	4	26.7	6	22.2	1	0.05
PAN150	3	20.0	4	0.2		
KK8					13	68.4
KK22					6	31.6
KK20					8	42.1

Interviewed farmers also narrated the benefits that they gained from the adoption of IPDM technologies (Table 3.3.7). These farmers further mentioned the effects of adopting the IPDM technologies on their households as i) the increased crop yield enabled them to have sufficient food at household level during periods of scarcity (59.6%, n=52), ii) improvement in general family health (53.9%, n=45), iii) general increases in household income (41.7%, n=48) and increased household food supply all year round (53.9%, n=13).

Table 3.3.7. Benefits from the use of selected IPDM technologies by farmers at Kisii project site in Nyanza province, Kenya.

IPDM technology	First benefit	Count	%	Second benefit	Count	%
Improved crop varieties	Leads to high yields	52	84.6	Effective against insects & diseases	52	15.4
Regular scouting	Effective against insects & diseases	51	58.8	Leads to high quality seeds	51	21.6
Timely weeding	Reduce soil nutrient competition	53	52.8	Leads to high yields	53	43.4
Use of botanicals	Cheap and easy to use	55	69.1	Effective against insects & diseases	55	25.5
Use of conventional chemicals	Effective against insects & diseases	55	85.5	Leads to high yields	55	14.5
Soil nutrient management	Improved soil fertility	55	63.6	Leads to high yields	55	16.4
Cropping system	Effective against insects & diseases	53	62.3	Leads to high yields	53	24.5
Timely harvesting	Effective against insects & diseases	53	60.4	Leads to high quality seeds	53	32.1
Drying, cleaning & sorting	Cheap and easy to use	55	41.8	Leads to high	55	25.5
Type of storage facility	Cheap and easy to use	55	65.5	Leads to high quality seeds	55	16.4
Storage pest control	Effective against insects & diseases	50	54.0	Cheap and easy to use	50	34.0

Other effects were increased in income controlled by women farmers and improvement in farmers to farmer and community relationships. The overall survey sample results are indicated in Figure 3.3.1. The negative changes experienced by farmers using the IPDM technologies include: increases in the amount of work done by women, increase in domestic fights between husbands and wives, and reduced prices of beans at the local market. The control of household income resulting from increased yield and income could be the main cause for the domestic fights. It is most likely that use of IPDM technologies that helped to increase yields in most households resulted in market price reduction as mentioned by the farmers. This observation shows the need for appropriate storage facilities and formation of credit and savings associations to enable farmers to store their grain and use loans for settling household needs until market prices become favorable. It is most likely that use of IPDM technologies that helped to increase yields in most households resulted in market price reduction as mentioned by the farmers. This observation shows the need for appropriate storage facilities and formation of credit and savings associations to enable farmers to store their grain and use loans for settling household needs until market prices become favorable.

The most effective bean IPDM technology dissemination channels that were mentioned by farmers include use of farmer research groups, setting up demonstration and learning plots, organizing and conducting field days, facilitating tours, conducting seminars and training workshops, using media particularly local radio, preparation and distribution of promotional materials (such as posters, leaflets, field guides, manuals), setting up village information centers (VICs), neighborhood and friendly exchanges, and consultations with researchers, extension and other service providers including NGOs, CBOs and stockists.

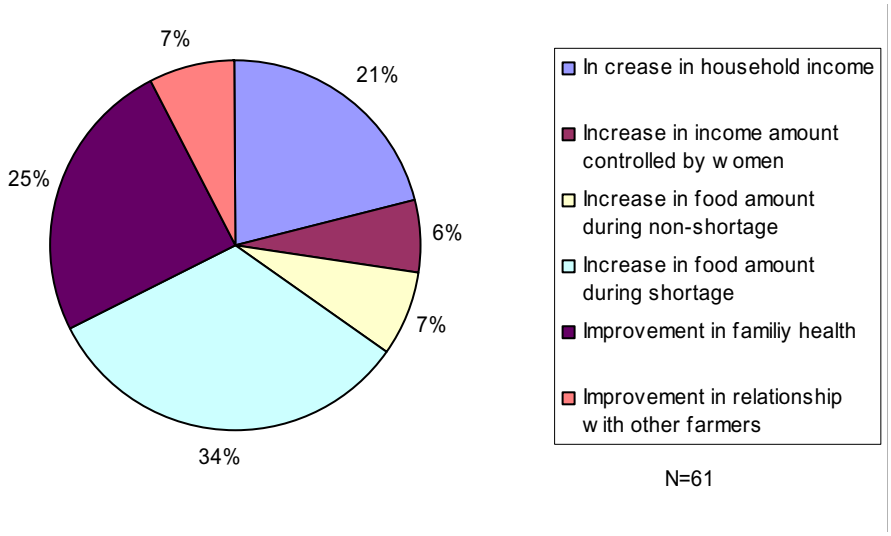

Figure 3.3.1. Positive changes perceived and mentioned by farmers from the use of IPDM technologies based on overall survey sample at Kisii site, Nyanza province in Kenya.

Northern (Hai district) and southern Tanzania: The studies in Hai district indicated that all IPDM research group members were knowledgeable on a number of practices and technologies. The source of information on such technologies varied between groups. Most information was obtained from the village extension officers – VEOs and the IPDM participating group members (Table 3.3.8).

Owners and users of the village information centers and collaborating partners have been sensitized to stock the centers with information materials on other aspects of community development according to users' demand. For example, publications on health issues (such as Malaria control, HIV/AIDS management and nutrition), education (adult education, general newsletters and magazines), agribusiness and general knowledge are found in some of the centers. Community members have been demanding different information materials after visiting the centers. Some members are keen to buy some of the materials for personal use. More farmer activity reports were printed and distributed to existing village information centers and primary school libraries in some of the project locations. New village information centers have been set up in northern Tanzania and Kisii project areas and more materials have been prepared and distributed to these VICs.

Table 3.3.8. Source of information on IPDM technologies for farmer research groups in Hai district, northern Tanzania.

Source	Percentage of farmers responding
Village extension officers	72
IPDM farmer groups	68
Demonstration and learning plots	55
Field days	46
Farmer friends	44
Researchers	36
Training workshops and seminars	32
Leaflets	28
Radio	26
Study tours/Cross visits	18
Non Governmental Organisations	10
Village information centres (VICs)	9
Market places	6
Copying from farmers	5
Traders	3

IPDM group members used different methods to disseminate information on IPDM technologies. The first approach was farmer research groups where group members organize meetings, attend seminars and workshops, set up learning and demonstration plots, monitor pests problem and control them, evaluate technology performance and organize field days and visits to share and disseminate information. Secondly, individual approach where each farmer talks to other farmers or demonstrate different technology options in their individual fields and train other farmers (Tables 3.3.9 & 3.3.10).

Table 3.3.9. Dissemination methods for IPDM technologies in Hai district, northern Tanzania

Dissemination method	IPDM groups		Participating farmers	
	Number	Percentage (n=12)	Number	Percentage (n=136)
Demonstration plots	7	58%	124	92%
Field day	4	33%	-	-
Radio	2	17%	-	-
Markets	1	8%	-	-
Religious places	3	25%	-	-
Village meetings	4	33%	-	-
Talk to other farmers	9	75%	118	87%
Leaflets	-	-	3	2%

Table 3.3.10. Dissemination methods/channels used by IPDM farmer groups segregated by gender in Hai district, northern Tanzania.

Dissemination method/channel	Male (n=54)	Female (n=60)	Total farmers (n=114)
Demonstrate on my own/group plot	42(78%)	38 (63%)	80 (70%)
Talk to other farmers	27(50%)	38(63%)	65 (57%)
Leaflets	10(18%)	3(5%)	13 (11%)

The groups mentioned other dissemination methods such as advertising in mosques or churches, talking to neighbors and relatives from within and outside the village, radio messages and participation in agricultural shows such as Nane Nane (National Farmers' Day). As the result of these efforts, the focus group discussion members estimated the number of farmers who are knowledgeable and use some of the IPDM technologies as it ranged from 40% to 80% depending on the age of the farmer research group.

Among the interviewed farmers in Hai district, 91% considered IPDM technologies to be advantageous in farm production, 86% reported increases in bean and maize production, 18% reported that the technologies were safe and relatively cheap compared to conventional chemical pesticides and fertilizers and 17% indicated that the technologies were easy to use. Farmers reported social economic benefits such as access to inputs, improved skills, information and new technologies that have helped them to increase farm production, resulting in increased household income and food security. The increased income was used to pay children school fees, purchase extra and better food, acquire better building materials and clothing, hired additional land for cultivation and some farmers were able to purchase livestock. Other benefits included reduced use of conventional chemical pesticides and fertilizers and links to new partners for information and services.

As farmer groups continued to be innovative in northern Tanzania, they have demanded to additional services and new technologies. In 2005 such farmers tested traditional and locally available organic fertilizers (animal manure, Minjingu Rock Phosphate and Minjingu Rock Phosphate + Solubilizers) on different bean varieties at Sanya Juu, Mogabiri and Makisoro villages in Hai, Tarime and in Arumeru.districts, respectively. A screen house trial on two of these varieties was set up at SARI in Arusha (Table 3.3.11 and Figures 3.3.2 – 3.3.4).

The data in the table shows the advantages of combining farmer yard manure and the rock phosphate where the moisture retention, nitrogen and other contents in the farm yard manure enhances the solubility of the rock. During a field day conducted by Sanya Juu bean IPM groups, farmers noted and were impressed by the vigorous stand and foliage retention of the fertilized bean plants (Figure 3.3.2), the pod and seed setting and the larger seed size compared to unfertilized plants. Bean stems and haulms are a source of quality livestock fodder that is used by all zero grazing livestock farmers in northern Tanzania.

Table 3.3.11. Bean grain yield response to on-farm organic fertilizer trials at Sanya Juu village, Hai district, northern Tanzania in March-July 2005 planting season.

Organic fertilizers	Mean bean grain yields on 3 varieties (tons/ha)		
	Lyamungu 90	**JESCA**	**Selian 94**
Unfertilized control	3.5d	4.1c	4.2d
Minjingu Rock Phosphate (MRP)	4.3c	5.8b	5.0c
Farm yard manure (FYM)	4.9b	7.1a	6.3b
Minjingu + Farm yard manure	6.8a	7.5a	7.1a
LSD at 0.05	0.006	0.007	0.006
CV (%)	13.8	14.3	12.8

Columns followed by the same letter are not significantly different (P=0.05)

Bean plants with Minjingu

Bean plants without Minjingu

Figure 3.3.2. Bean plants response to Minjingu Rock Phosphate (MRP) application at Sanya Juu village, Hai district in northern Tanzania during March-July planting season in 2005.

Links with Farm Inputs Promotions Africa Ltd- FIPS (an NGO) and Minjingu Mines & Fertiliser Ltd (private company) has helped to create awareness with farmers in Arumeru, Moshi and Tarime districts in northern Tanzania. Bean farmer groups in the 3 districts experimented with Minjingu Rock Phosphate (MRP) that has been fortified with Ca at 16%, K9%, N7% and S 5% help in the solubility of the rock and enable it to become available to short duration and fast growing crops including beans and vegetables. The company further agreed to pack the fertilizer in small packets (1kg) for distribution to bean farmer groups for demonstrations in their home gardens.

More than 200 samples (@ Ikg) of the fertilizer mixture were distributed to farmers in the three districts. Experiments in Arumeru and Tarime were conducted on bush beans while in Moshi it was tested on bush and climbing beans. The currently ending short rain season was characterized by erratic rainfall (Moshi and Tarime) and irrigation water shortages (Arumeru and Moshi) but generally farmers were impressed with the vigorous bean plant growth, larger biomass and the comparatively low severity of bean stem

maggots damage (Figure 3.3.3), improved tolerance to water stress, higher grain yield (1.5-3.0 times) compared to the unfertilized plants (Figure 3.3.4), larger seed size, higher number of seeds per pod and plot (such as 260 compared to 80 seeds per farmer plot at Makisoro village in Arumeru).

Figure 3.3.3. Response of bean plants to Minjingu Mazao at Mogabiri in Tarime, northern Tanzania.

Improved bean seed testing and dissemination has cut across Uganda, Kenya, Tanzania and Malawi as the one of the major activities in IPDM technology promotion. In Uganda and Kenya it addressed the root rot, bean stem maggots and soil fertility constraints while bean stem maggots, bean foliage beetles, aphids and soil fertility were the main focus in Maklawi and Tanzania. In the southern highlands of Tanzania for example, the Uyole Agricultural Research Institute (ARI Uyole) sold and distributed over 3000 kg of improved bean seed to farmers in collaboration with partners especially the Evangelical Lutheran Church of Tanzania (ELCT) in Mbozi district and the extension services in all other districts (Table 3.3.12).

Table 3.3.12. Improved bean seed distribution and variety demonstrations in Mbozi district, southern Tanzania during 2005/06 planting season.

Bean variety/line	Amount of seed (kg) purchased and distributed	Number of varieties/lines in on-farm trials	Key partners
Uyole 96	2050	-	Farmers (19 women, 48 men) in 13 villages, ELCT, Extension
Uyole 98	350	-	ARI Uyole, Extension
Uyole 94	100	-	ELCT Mlowo congregation
Wanja (G22501)	200	-	Farmers, Extension
Urafiki	30	-	Farmers, Extension
Calima lines	-	6	ARI Uyole, Farmers, Extension personnel
DRK lines	-	3	Farmers in 4 villages (26 women, 22 men)
Sugar lines	-	2	ELCT, Farmers, extension
Yellow type	-	1	Farmers, Extension

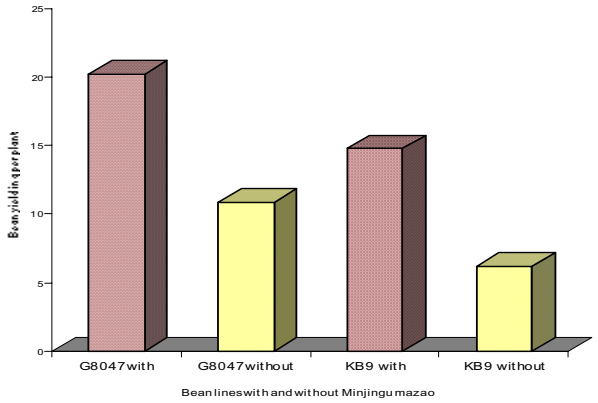

Figure 3.3.4. Grain yield response to Minjingu mazao fertilizer in screen house experiment.

Activity 3.4. Capacity building

Contributors: Staff members of PE-1 and partners

Table 3.4.1. List of students supervised in 2005.

BSc Thesis

Name	Supervisor	University	Title
Javier Francisco Abello C. (Feb. 2004 – Aug. 2005)	Segenet Kelemu (CIAT) Celsa García (Universidad Nacional)	Universidad Nacional de Colombia, Bogotá	Transformación del hongo *Acremonium implicatum* (J. Gilma & E.V. Abbott) W. Gams, con el gen que codifica para la proteina verde fluorescente (*gfp*), mediada por *Agrobacterium tumefaciens* Smith & Townsend.
Jorge Alejandro Corredor (Aug. 2004 – March 2005)	Elizabeth Álvarez (CIAT) Gerardo Martínez (Universidad de Caldas)	Universidad de Caldas	Evaluation of the association of morphological and biochemical characteristics of cassava (*Manihot esculenta* Crantz) roots with resistance to root caused by *Phytophthora tropicalis* and postharvest physiological deterioration.
José Luis Claros (Feb. 2005 – Sept. 2005)	Elizabeth Álvarez (CIAT) Martín Fregene (CIAT) Jaime Eduardo Muñoz (Universidad Nacional)	Universidad Nacional de Colombia	Identifying resistance gene analogs associated with bacterial wilt in the ACR cassava family

Table 3.4.1. List of students supervised in 2005, cont´d

BSc Thesis

Name	Supervisor	University	Title
Eduardo Gómez Aug 2004 – Sept.2005	Elizabeth Álvarez (CIAT) Clemencia Forero de La Rotta (Universidad Javeriana)	Universidad Pontificia Javeriana, Bogotá	Identifying and characterizing isolates of *Ralstonia solanacearum* obtained from areas affected by bacterial wilt of plantain in Colombia
Carolina Buitrago Aya (Mar 2004 – Oct. 2005)	Andreas Gaigl (CIAT) James Montoya (Universidad del Valle)	Universidad del Valle, Colombia	Evaluación de la patogenicidad y virulencia de *Bacillus popilliae* Dutky sobre larvas de segundo instar de *Phyllophaga menetriesi* Blanchard (Coleoptera: Melolonthinae)
Anyimilehidi Mazo Vargas	James Montoya (Univalle) Anthony Bellotti (CIAT)	Universidad del Valle, Colombia	Efecto del algodón Bollgard® (Bt) sobre la diversidad y abundancia de artrópodos del suelo durante su segundo año en el Valle del Cauca
Luz Adriana Mesa Becerra (Oct. 2005 – Oct. 2006)	Elizabeth Alvarez (CIAT) Julio C. Torres (Universidad del Quindío)	Universidad del Quindío, Colombia	Alternativas de manejo de Moko de plátano en focos afectados (Finca La Florida)
Andrés Rincón (Jul. 2005 – Dec. 2005)	Elizabeth Alvarez (CIAT) Andreas Gaigl (CIAT) Inés María Ulloa (Universidad del Valle)	Universidad del Valle, Colombia	Evaluacion exante de efecto económico y financiero para el manejo integrado de suelos, plagas y enfermedades de la yuca en las regiones del Cauca y Llanos Orientales
Lorena Cortes (March 2004- May 2005)	George Mahuku (CIAT)	Universidad del Valle	Efecto de las diferentes fuentes de abono verde en el suelo sobre el manejo de hongos causantes de pudriciones en el frijol (*Phaseolus vulgaris*, L. Fabaceae).
Eliana del Pilar Macea (Aug. 2005-Aug 2006)	Anthony Bellotti (CIAT) Jaime Montoya Lerma	Universidad del Valle	Identificación de marcadores moleculares asociados con la caracteristica de resistencia al acaro verde en yuca
Gabriel A. Torres Londoño (Oct. 2005 – Oct. 2006)	Anthony Bellotti (CIAT) Gerardo Martínez López, (Universidad de Caldas)	Universidad de Caldas, Colombia	Evaluación de *Sogatella kolophon* (Kirkaldy) y *Empohasca bispinata* (Davidson & Delong) como posibles especies vectoras de la Enfermedad del Cuero de Sapo en yuca
Víctor Hugo Treviño Henao (Oct. 2005 – Oct. 2006)	Elizabeth Alvarez (CIAT) Julio César Torres Arbeláez (Universidad del Quindío	Universidad del Quindío, Colombia	Alternativas de manejo de Moko de plátano en focos afectados (Finca La Manigua)

Table 3.4.1. List of students supervised in 2005, cont´d

Name	Supervisor	University	Title
BSc Thesis			
Marcelo Vargas (Feb. 2005 – Feb. 2006)	Elizabeth Alvarez (CIAT) Henry Toro (Universidad de Caldas)	Universidad de Caldas, Colombia	Evaluación de prácticas ecológicas de manejo de suelo en focos afectados por Moko (*Ralstonia solanacearum*) en un cultivo de plátano (Finca La Guaira) en el departamento del Quindío
Omar Zuluaga (Feb. 2005 – Feb. 2006)	Elizabeth Alvarez (CIAT) Henry Toro (Universidad de Caldas)	Universidad de Caldas, Colombia	Evaluación de prácticas ecológicas de manejo de suelo en focos afectados por Moko (*Ralstonia solanacearum)* en un cultivo de plátano (Finca Cataluña) en el departamento del Quindío
María Fernanda Miller	Cesar Cardona (CIAT) James Montoya (Univ. del Valle)	Universidad del Valle, Cali, Colombia	Sub-lethal effects of a resistant *Brachiaria* hybrid on the demography of the spittlebug Zulia *carbonaria* (Lallemand) (Homoptera: Cercopidae)
Sandra Jimena Valencia	Cesar Cardona (CIAT) Magnolia Cano (Univ. Nacional Palmira, Colombia)	Universidad Nacional Palmira, Colombia	Sub-lethal effects of antibiosis on the demography of *Zabrotes subfasciatus* and *Acanthoscelides obtectus*, storage pests of beans.
David Pulgarin	Fernando Correa (CIAT) Gustavo Adolfo Garcia Henao (Univ. de Antioquia, Medellín, Colombia)	Univ. de Antioquia, Medellín, Colombia	Caracterización de genes de resistencia a *Pyricularia grisea* (Sacc.) en variedades de Arroz de America Latina y del Caribe

MSc Thesis

Name	Supervisor	University	Title
María Elena Cuellar Jiménez (Feb. 2004 – March 2006)	Francisco Morales (CIAT) James Montoya (Universidad del Valle)	Universidad del Valle, Colombia	El arrugamiento foliar del frijol: Transmisión por la mosca blanca (Gennadius) (Homoptera: Aleyrodidae), búsqueda de fuentes de resistencia en *Phaseolus vulgaris* L. y epidemiología
Miriam Karlsson (Dec. 2004 – Aug. 2005)	Anthony Bellotti, Claudia Holguín (CIAT) James Montoya (Univalle) Sven Axel Svensson (Swedish Univ. of Agric. Science)	Swedish University of Agricultural Science	Control de mosca blanca (*Aleurotrachelus socialis* Bondar) con insecticidas orgánicos en yuca (*Manihot esculenta* Crantz)
Sophia Komba (Jun. 2003 – Dec. 2005)	Susan Kaaria, Eli Minja (CIAT) Felician Mutasa (Open University of Tanzania)	Open University of Tanzania and New Hampshire University (USA)	Social economic benefits of IPDM technologies to bean farming communities in Hai district, northern Tanzania

Table 3.4.1. List of students supervised in 2005, cont´d

MSc Thesis

Name	Supervisor	University	Title
Juan Fernando Mejía (Oct. 2005 – Oct. 2007)	Elizabeth Alvarez (CIAT) Martín Fregene (CIAT) Jaime E. Muñoz (Universidad Nacional)	Universidad Nacional, Colombia	Identificación de genes análogos de resistencia asociados con superalargamiento en la familia ACR de yuca
Maria Antonia Henriquez (Jan.2003 – Aug.2005)	George Mahuku (CIAT) Hernando Ramirez (Universidad Nacional de Colombia, Palmira)	Universidad Nacional de Colombia, Palmira	ESTs para entender la interacción entre genotipos del frijol común (*Phaseolus vulgaris*) y *Phaeoisariopsis griseola*, el agente causal de la mancha angular
Walter Ocimati (Sep. 2004 – Dec. 2005)	Robin Buruchara (CIAT) Geoffrey Tusiime (Makerere University)	Makerere University, Uganda	Effects of different management practices against root rots on major crops in the bean-based cropping system of south-western Uganda
Augustine Musoni	Robin Buruchara (CIAT)	University of Nairobi	Inheritance of fusarium wilt (*F. oxysporum* f.sp. *phaseoli*) and selection for multiple disease resistant and marketable climbing bean varieties
Carlos Alberto Ortega-Ojeda (Jan. 2004 – Dec. 2005)	Andreas Gaigl (CIAT) César Falconi (Escuela Politécnica del Ejército)	Escuela Politécnica del Ejército; Centro de Posgrado (Ubicado en Sangolquí-Distrito Metropolitano de Quito)	Estudios metodológicos para evaluar el impacto económico de escarabajos Melolonthidae (Insecta: Coleoptera) en cultivos tropicales
Leopoldo Serrano	Francisco Morales (CIAT)	Universidad de El Salvador	Caracterización de biotipos de la mosca blanca
Ana Karina Martinez	Francisco Morales (CIAT) F. Alirio Vallejo (Univ. Nacional Palmira)	Universidad Nacional Palmira, Colombia	Caracterización del *begomovirus* y evaluación de líneas como fuente para la producción del tomate con resistencia a *begomovirus* en el Valle del Cauca
Maritza Cuervo	Lee Calvert (CIAT) Edgar Ivan Estrada (Univ. Nacional Palmira)	Universidad Nacional Palmira, Colombia	Caracterización molecular de algunos aislamientos del virus del cuero de sapo de la yuca recolectados en diferentes zonas de Colombia
Fernando Lopez	Cesar Cardona (CIAT) James Montoya (Univ. del Valle)	Universidad del Valle, Cali, Colombia	Tolerance to adult feeding damage as a component of resistance to *Aeneolamia varia* in *Brachiaria* spp.

Table 3.4.1. List of students supervised in 2005, cont´d

Name	Supervisor	University	Title
Ulises Castro	Cesar Cardona (CIAT) Jorge Vera graciano (Universidad of Chapingo, Mexico) Ramón Garza García (INIFAP)	Universidad of Chapingo, Mexico	Mechanisms of resistance to *Aeneolamia Albofasciata* and *Prosapia simulans* in *Brachiaria* spp.
Alejandro Pabón	Cesar Cardona (CIAT) Evaldo Ferreira Vilela (Departamento de Biologia Animal/UFV)	Universidad of Viçosa, Brazil	Mechanisms of resistance to *Deois incompleta, D. Schah* and *Notozulia entreriana* in *Brachiaria* spp.
Paola Sotelo	Cesar Cardona (CIAT) Ariel Gutierrez (Univ. Nacional Palmira, Colombia)	Universidad Nacional Palmira, Colombia	Inheritance of crumpled leaf virus in snap beans

Ph. D. Thesis

Name	Supervisor	University	Title
Clare Mukankusi (Sep. 2003 – Aug. 2007)	Robin Buruchara (CIAT) Rob Melis (University of KwaZulu-Natal)	University of Kwazulu-Natal, Pietermaritzburg, RSA	Breeding beans (*Phaseolus vulgaris* L.) for resistance to Fusarium root rot (*Fusarium solani* f.sp. *phaseoli*) and large seed size in Uganda
Virginia Gichuru (Sept. 2005 – Aug. 2006)	Robin Buruchara (CIAT) Patrick Okori (Makerere University)	Makerere University, Uganda	Symptomatology and characterization of *Pythium* spp. of major crops in a bean based cropping system in south-western Uganda
Reuben Otsyula	Robin Buruchara (CIAT)	Makerere University, Uganda	Study of inheritance and development of root rot (Pythium) resistant varieties using marker assisted selection in common beans
Arturo Carabalí (April 2005- April 2008)	Anthony Bellotti (CIAT) James Montoya Lerma (Universidad del Valle)	Universidad del Valle, Colombia	Caracterización de la Resistencia a la Mosca Blanca *Aleurotrachelus socialis* Bondar en Especies Silvestres de *Manihot* y Marcadores Moleculares Asociados
Enrique Bravo	Francisco Morales (CIAT)	Universidad del Valle, Cali, Colombia	Secuencia Nuclotídica y estructura del genoma del virus del mosaico común del frijol cepa NL4
Helena Reichel	Francisco Morales (CIAT)	University of Gembloux, Belgium	Caracterización del virus flamentoso del platano en Colombia, Filipinas y Africa
Gloria Santana	Francisco Morales (CIAT)	Universidad Nacional Palmira, Colombia	Resistance to bean common mosaic virus

Activity 3.5. Training and consultancy services offered during 2005

Contributors: Members of PE-1

Event	Date	Organizer/ Place	Participants	Received by
Diagnostic and identification of bacterial and fungal pathogens	Sep 26-Oct. 7	CIAT-Cassava Pathology/ Palmira	2	Isola Robleto y Ana María Blanco, MAGFOR, Nicaragua
Training in nematodes, entomopathogenic fungi and bacteria	Jul. 18.-Nov. 2	CIAT – cassava entom. / Palmira	1	Sandra Victoria Mena
Conservation and handling of entomopathogenic fungi	Jul.13-Oct 30	CIAT - cassava entom./ Palmira	1	Ifigenia Hurtado
Raising and handling of "chinche" and "galleria"	Jul. 18-28	CIAT- cassava entom. / Palmira	1	Eliécer Vivas, Technician LST S.A., Bogotá
Risk evaluation of genetically modified organisms (GMOs): Bt cotton case in the Valle del Cauca, Colombia.	Jun. 27	ICA/Tulua	25	Agricultural and extensión specialists
Pathological, entomological problems, integrated crop management, and knowledge on nitrifying bacteria	May 20	CIAT- cassava entom / Palmira	30	Students in VIII Semester of Economic Entomology and forage protection, Agronomy program, Fac. Ciencias Agropecuarias, Universidad de Caldas, Colombia
Training in storage and conservation of entomopathogenic fungi	Apr. 28	CIAT/Palmira	14	Students, Universidad de Córdoba, Colombia
International course of modern production, processing and utilization systems of cassava in Latin America and the Caribbean, integrated management of whitefly, horn worm and utilization of *baculovirus.* Biological control of burrower bug with nematodes	Apr. 18-27	CIAT- Clayuca/ Palmira	34	Professionals and technicians from Latin America and the Caribbean
First workshop on "Basic Taxonomy and Overview of Collembola"	Apr.l 11-15	CIAT-Cornell University project/Palmira	11	Students from Universidad del Valle, Colombia
Biological control, soil quality indicators, molecular markers of beans	Apr. 6	CIAT	25	Students from Universidad Nariño, Colombia
Trainning in procedures for risk evaluation of genetically modified organisms (GMOs)	Mar. 18-Apr. 8	CoaCol/Palmira	1	Luisa Fernanda Bermúdez, Universidad Nacional de Colombia

Activity 3.5. Training and consultancy, (cont´d)

Event	Date	Organizer/ Place	Participants	Received by
Advances in diagnosis and integrated management of bacterial wilt of banana and plantain	Mar.10–11, 2005	CIAT, Palmira		Martha Cecilia Castaño, Ana Lucía Bejarano, Angela María Arango, Marco Fabián Flórez, and Jefferson Rubiano, professionals and technicians from ICA–Section Quindío
First update on sustainable production of maize	Feb. 24	ICA, Corpoica, Industrias del Maiz, Casa Toro, Del Campo/ Palmira	200	Students, extensión and agricultural specialists
Training in overview of cassava crop management	Feb. 8-10	CIAT- cassava entom, Palmira	1	José M. Lugo Pérez, Caribbean Best, Costa Rica
Biodiversity of collembolas in silvo pastoral and agricultural production systems in the tropical dry forest	Feb. 7-11	CIAT/Palmira	1	Claudia Lisana Guzmán del Río, Universidad del Tolima, Colombia
Advances in research for managing bacterial wilt of plantain	Oct. 26	ICA-Tulua	19	Technical personnel from ICA Tulua, Colombia
Advances in research on managing bacterial wilt in Musaceae	Sep. 29	ICA- Palmira	18	Technical personnel from ICA Palmira
Workshop on integrated management of cassava pests and diseases with emphasis on biological control and production costs	Sep. 9		23	23 participants from Yopal, Colombia
Managing cassava diseases	Aug. 18, 19	CIAT, Palmira		Ana Elizabeth Diaz, CORPOICA; Juan Jose Font and Jorge Fuentes, Guatemala
Seminar on “Advances in the Search for Alternatives in Managing Bacterial Wilt of Musaceae	Aug 18	Sevilla, Valle del Cauca	30	Farmers and technicians
Advances in the search for alternatives in managing bacterial wilt of plantain: national seminar on plantain pests and diseases	Jul 18-19		300	Farmers, technicians, and students from Armenia, Colombia
Workshop on the management of cassava diseases, using clean strategies	Jul. 5	CIAT, Palmira	11	Farmers form Piendamó, Cauca

Activity 3.5. Training and consultancy, (cont´d)

Event	Date	Organizer/ Place	Participants	Received by
Managing bacterial wilt of plantain in Quindio, Colombia	Jul 1-31	CIAT, Palmira	2	Marcelo Vargas and Omar Zuluaga, Universidad de Caldas, Colombia
Development of strategies for managing bacterial wilt of plantain	15 Jul–15 Dec, 2005	CIAT, Palmira	1	Juan Pablo Castle, Universidad de Caldas, Colombia
Application of biofungicides in grape disease control: evaluation of the inhibitory minimum concentration of several products on *Ralstonia solanacearum*	May 23– Jul. 21, 2005	CIAT, Palmira	1	Mathilde Ouevrard, Institut National d'Horticulture, France
Training on the agronomic management of snap and dry beans and integrated disease management.	Nov. 17-19	Bogota	65	Technicians
Course on cassava bacterial blight	5 May	CIAT/U. Nacional de Palmira	2	Johny Arlet Pineda Calle and Claudia Patricia Gonzales Students, U. Nacional de Palmira, Colombia
PCR methods to detect *Ralstonia solanacearum* in soil samples collected from a commercial plot of plantain in the Ariari Region	Apr 30.- May 13	CIAT, Palmira	1	Sandra Milena Rodríguez CORPOICA Villavicencio
Identifying isolates of *Ralstonia solanacearum* through specific PCR, using primers	Apr. 8- May 5	CIAT, Palmira	1	Sandra Milena Rodríguez CORPOICA Villavicencio
Symptoms and management of cassava frogskin disease	Mar. 28	CIAT	43	Farmers form Armenia
Detecting phytoplasmas associated with frogskin disease of cassava	Nov 2004- Nov 2005	CIAT, Palmira	2	Adriana Arenas and Diana López, students, Universidad del Valle, Colombia
Innovation Histories of the Adoption of Bean Varieties		CIAT, Kampala, Uganda	16	
Strengthening Capacity for Participatory Monitoring and Evaluation for the National Bean Programmes in ECABREN Partner Countries, Nairobi, Kenya	Mar14 - 18	CIAT	35	
Biofortification Project Planning meeting	Mar19	Nairobi, Kenya	13	
Planning of bean research	Mar 30-31	Cameroon	15	
Participatory Plant Breeding Monitoring Tour	Apr1-12	CIAT/ Ethiopia	12	

Activity 3.5. Training and consultancy, (cont´d)

Event	Date	Organizer/ Place	Participants	Received by
Ninth Annual Meeting of the PABRA Steering Committee	16 - 18 May	Arusha, Tanzania	17	
Ouru Masawa farmer field day	18 May	Kenya	107	Farmers
Ethiopia Bean Seed Impacts Partner Meetin6	20 - 21 April			
Workshop on Strengthening Common Bean Seed System in Eastern Ethiopia	May 30	Jima, Ethiopia	15	
Breeder's course on making crosses	8-11 June	Uganda	20	
Breeder's Course and Field Visits	June 7 - 18,	Uganda/ Rwanda/ Kenya	17	
Training of enumerators at Oyugis for IPDM uptake surveys in Kisii site	25 -26 August	Kenya	16	
Reviewing PABRA Regional Strategy for IPDM & INM in Beans	Oct 31- Nov 3	Kampala, Uganda	33	
Marker Assisted Selection in Plant Breeding: Principles and practices	3 - 7 October	Kawanda, Uganda	13	
To facilitate a farmer exchange visit and conduct IPM farmer group participatory training workshop at Tarime for VicRes Project participants	22-26 Nov	Tarime, Tanzania and Kisii, Kenya	25	
Tarime farmers' learning visit to Ouru Masawa	24 Nov	Tanzania	26	Farmers
Training of Tarime farmers on bean IPDM and participatory group research approach	25 Nov	Tanzania	27	Farmers
How to measure parasitism in whiteflies	Feb. – Mar.	CIAT	120	Farmers
Biology and ecology of whiteflies	23 Feb.	CIAT	30	Technical assistants and farmers
Training in sampling methods	22 April	CIAT	8	Students, Univ. Nacional /Palmira
Biology and ecology of whiteflies	4 May	CIAT	20	MSc. students, Univ. Nacional / Medellín
Biology and ecology of whiteflies	19 May	CIAT	35	Students, Univ. de Caldas- Manizales
Sampling insects, biology of whiteflies	June	CIAT	65	Students, Univ. Nacional /Palmira
Biology and ecology of whiteflies	Sept.	CIAT	2	Students, Univ. de Caldas- Manizales

Activity 3.5. Training and consultancy, (cont´d)

Event	Date	Organizer/ Place	Participants	Received by
Management of whiteflies	12 October	CIAT	19	Students, Univ. Nacional /Palmira
Management of whiteflies	10 –22 Oct.	CIAT	1	Vladimir Lino Collaborator from PROINPA
Sampling insects, biology of whiteflies	8 Nov.	CIAT	27	Students, Univ. Nacional /Palmira
Viral Diseases of High Value Crops	23-25 Nov.	CIAT	20	Personnel from Semillas Arroyave

Activitiy 3.6. Conferences, Workshops, Meetings Attended by one or more staff of PE-1 project

Staff Member	Date	Place	Event
Segenet Kelemu	4-6 April	Nairobi, Kenya	Sub-Saharan (SSA) regional design meeting for the International Assessment of Agricultural Science and Technology for Development (IAASTD)
	21-24 February	Aleppo, Syria	System-wide Program-IPM (SP-IPM) committee meeting, ICARDA
	30 July-4 August	Austin, U.S.A	American Phytopathological Society meeting
	6-10 July	Ravello, Italy	Internacional consortium on agricultural biotechnology research (ICABR) conferences
	25-29 September	El Batan, Mexico	First level leadership development program (FLDP), CIMMYT
Elizabeth Alvarez	10-13 May	Angers, France.	5th ISTA–SHC Seed Health Symposium
	14-17 May	Lyon, france	Ecole National Veterinaire de Lyon.
	28 August – Sept 2	Manizales, Colombia	II Seminar on Production, Commercialization e Industrialization of Plantain
	4-9 October	Bogotá, Colombia	XXVI ASCOLFI Congress
	11-13 May	Palmira, Colombia	IX Congress of the Colombian Association of Plant Breeding and Crop Rots, CORPOICA
	26 August	Palmira, Colombia	Workshop on the "State of the Art in the Agricultural Prediction Models", Corporación BIOTEC, SAG, CENICAÑA, CIAT
	29 July	Palmira Colombia	Workshop on "Participatory selection of soursop planting material: an analysis of advantages and implications," Corporación BIOTEC, CIAT, PROFRUTALES Ltda, COLCIENCIAS
Andreas Gaigl	11-18 April	Manaus, Brazil	TSBF Annual Meeting
	25-29 June	Quito, Ecuador	Meeting with Wilson Vasquez (INIAP) and Trevor Jackson (AgResearch, NZ), set up of proposal for NZAID

Activitiy 3.6. Conferences, Workshops, (cont´d)

Staff Member	Date	Place	Event
	27 July	Ibague, Colombia	SOCOLEN Annual Congress
	7-8 December	Quito, Ecuador	Defense of MSc thesis by Carlos Alberto Ortega, MSc Student
Cesar Cardona	13-18 November	Brazil	Review of work on host plant resistance to spittlebug (University of Viçosa)
	26 February- 2 March	London, UK	Planning meeting of the Whitefly Project at DFID Headquarters
	20-24 October	Bolivia	Planning meeting of Whitefly Project activities in Bolivia. With Proinpa, the national collaborator.
	March, August	Chota, Ecuador	Visit whitefly management trials
Eli Minja	March 14-18	Nairobi, Kenya	CIAT PABRA PM&E workshop
	31 May – 4 June	Uganda	Africa Staff meeting and BOT review
	26-30 June	Nairobi, Kenya	ASARECA Concept Note proposal with Kenya national scientists
	July 13-15	Nairobi and Kirinyaga, Kenya	To participate in Farm Inputs Promotion Africa (FIPS, an NGO) small package inputs methodology field day and meet with DFID-CPP Deputy Manager and other CPP Project Leaders based in Kenya and Tanzania
	October 16-21	Kisii, Kenya and Tarime, Tanzania	Vicres Project annual monitoring exercise
	Oct 30-Nov 04	Mukono, Uganda	CIAT PABRA INM&IPM framework workshop
	Nov 22-26	Tarime, Tanzania and Kisii, Kenya	To facilitate a farmer exchange visit and conduct IPM farmer group participatory training workshop at Tarime for VicRes Project participants
Robin Buruchara	12-15 January	Accra	SSA_CP Challenge Program meeting
	15-19 January	Kigali, Rwanda	Interviews for ATDT/ERI position
	23-28 January	Nairobi, Kenya	Attend the RF workshop of RF grantees
	27 Feb-4 March	Cameroon	Planning of Bean Research
	7-8 March	Butare, Rwanda	Seasonal planning meeting
	14-18 March	Nairobi, Kenya	PABRA PME workshop
	4- 6 April	Nairobi, Kenya	SSA Design Team Meeting
	24- 30 April	Kigali, Rwanda	SSA-CP Kivu Pilot Site Stakeholders meeting
	15-19 May	Arusha, Tanzania	PABRA SC Meeting
	1 - 4 June	Kampala, Uganda	Africa Staff Meeting and CIAT BOT Meeting
	6- 10 June	Entebbe, Uganda	FARA General Assembly

Activitiy 3.6. Conferences, Workshops, (cont´d)

Staff Member	Date	Place	Event
	22-24 June	Butare, Rwanda	Participate in the CGS proposal development for the GLR team
	28-30 June	Nairobi, Kenya	KEPHIS for a meeting to develop a proposal outline on "Good Seed Initiative"
	July 24 to 28	Washington, USA	Discuss the REU proposal at Harvest Plus IFPRI
	July 29- 6 August	Austin, USA	American Pytopathological Society meeting
	29 August -1 Sept	Entebbe, Uganda	SSACP, Lake Kivu Management Committee Preparatory meeting
	13 – 15 Sept	Nairobi, Kenya	ILRI, Harmonization of Medium Term Plans of CG in ECA.
	19 – 20 Sept	Gisenyi, Rwanda.	SSA-CP Lake Kivu Management Committee meeting
	21 – 23 Sept	Bukavu,, DRC	Planning meeting of the DGDC legume project.
	26 – 30 Sept	Kampala, Uganda	Bean REU proposal writing
	10 -13 October	Nairobi, Kenya	SSA-CP Concept Note Workshop
	25 – 28 October	Kabale, Rwanda, Goma	Accompany the EU Review mission of the SSACP
	31 Oct – 4 Nov	Kampala, Uganda	IPDM and INM Workshop
	6 – 13 Nov	Cameroon	Visit be program activities
	22 – 27 Nov, 2005	Kigali, Rwanda	Kigali, SSACP - Lake Kivu Log Frame Dev meeting
	1 Dec	Kampala, Uganda	SSACP, Lake Kivu, Management Committee Meeting.
George Mahuku	24-27 January	Nairobi, Kenya	Participate in the 2nd general meeting of the Rockefeller Foundation-supported program Biotechnology, Breeding and Seed systems for African Crops.
	1-5 May	Panama City, Panama	Attend the PCCMCA meeting
	29 May - 20 June	Kampala, Uganda	Attend the board meeting in Uganda, followed by field visits to Uganda, Rwanda, DRC and Kenya
	29 July - 4 August	Austin, U.S.A.	Attend the APS annual meeting
	23 September - 1 October	Kunming, China	Visiting Yunnan Academy of Agricultural Sciences in the Yunnan province of the People's Republic of China
	28 November - 10 December	Harare, Zimbabwe	Proposal development for the SADC pilot site of the SSA-CP
	17-19 November	Bogotá, Colombia	Workshop to train technicians (65) on the agronomic management of snap and dry beans and integrated disease management.
Anthony Bellotti	27 February- 2 March	London, U.K.	Tropical Whitefly project DFID
	6 - 10 June	Gainesville, USA	Seminar/Tropical Entomology Course on Cassava Pests; Discuss cooperation on invasive

Activitiy 3.6. Conferences, Workshops, (cont´d)

Staff Member	Date	Place	Event
			species, UF, Homestead, FL.
	10 - 16 September	Davos, Switzerland	International Symposium Biologicla Control
	26 October - 6 November	Campo Grande and Cruz das Almas, Brazil/ Fort Lauderdale, USA	CNPMF/EMBRAPA on Generation Challenge Program, Bahia Branca. Attend Entomology Society of America 2005 Congress; poster presentation.
	7-8 December	Quito, Ecuador	MSc Thesis defense, Carlos Alberto Ortega, Universidad Polytecnico
Francisco Morales	3 - 8 April	Lima, Peru	IX International Plant Virus Epidemiology Symposium
	19-22 April	Cordoba, Argentina	XIII Phytopathology Congress and the 3rd Workshop of the Phytopathology Association
Fernando Correa	4-6 April	Costa Rica	FLAR meeting
	18-24 April	Argentina	ALF meeting and FONTAGRO project
	12-19 June	USA	RiceCap meeting (Rhizoctonia) and workshop on MAS
	22-26 August	Panama	Observation of rice diseases. Project development
	16-28 November	Philippines	Rice Genomics Symposium and IRRI
	7-9 December	Venezuela	Redbio Conference

Activity 3.7. List of visitors to the various research activities of PE-1 project

CIAT – Palmira, Colombia

Name	Institution	Date
Dr. Jairo Osorio	CORPOICA, Colombia	26 January, 20 September
Dr. Lisette Staal	Internacional Program, University of Florida, USA	27 January
Mr.. José M. Lugo Pérez	Caribbean Best, Costa Rica	February 8-10
Dr. Milton Salazar	Subgerente Dupont	February 15
Mr. Fabio Clavijo	Ministry of Agriculture and Rural development	February 18
Mr. Gustavo Leon Villa	Biotropical S. A., Colombia	March 01
Dr. Masayoshi Saito	Liaison Officer of CGIAR Secretariat, outposted from JIRCAS	March 8 - 10
Ms. Kayo Fujita	Research coordinator, International Research Division, MAFF Research Council Secretariat of Japan	March 8 - 10
Mr. Hiroya Okamoto	Second Secretary, Embassy of Japan in Bogotá	March 8 - 10
Dr. Tatsuro Katsuyama	Director of International Research Division, MAFF Research Council Secretariat of Japan	March 8 - 10

Activity 3.7. List of visitors, (cont´d)

Name	Institution	Date
Dr. Yusaku Uga	Rice Genetist	March 8-12
Dr. Aaron Zazueta	Senior Specialist in Monitoring & Evaluating Global Environment	
Ing. Martha C. Castaño, Ana L. Bejarano, Angela M. Arango, Marco F. Flórez and Jeferson Rubiano	ICA – Seccional Quindio, Colombia	March 10-11
43 Farmers from Armenia, Colombia		March 28
Mr. Germán Arteaga M.	Universidad de Nariño, Pasto, Colombia	April 4
Ms. Chrissie Rey	University of the Witwatersrand, Johannesburg, South África	April 5 – 6
Prof. Mauricio Salazar Yepes and 14 students	Universidad Nacional of Colombia, Palmira, Colombia	April 7
Ms. Lilian Martinez and 26 students	Colegio Bolivar, Cali, Colombia	April 7
Prof. Henry Toro Lopez and 4 students	Universidad de Caldas, Colombia	April 20
Prof. James Montoya and 8 students	Universidad del Valle, Colombia	April 22
Drs. Santiago Perry, Andres Laignelet	CORPOICA	April 22
Mr. Johny A. Pineda and Ms. Claudia P. González	Universidad Nacional de Colombia, Palmira	May 5
11 Students	Universidad del Valle, Colombia	May 11
Dr. Lucia Vaccaro	Facultad de Agronomía, Universidad Central de Venezuela	May 16 - 20
Dr. Fanny Restrepo	CENIFLORES, Colombia	May 18
Prof. Henry Toro Lopez and 30 students	Universidad de Caldas, Colombia	May 19
Dr. Humberto Rios	Coordinator, Fitomejoramiento Participativo, Nacional Institute of Agricultural Sciences, Cuba	May 19-26
Dr. Silverio González	Coordinator, Cadenas Productivas de Plátano, FEDEPLATANO, Colombia	May 23
Prof. Luis Jairo Silva and 25 students	Universidad Dist. Francisco de Caldas	May 25
Prof. Hernando Delgado and 28 Students	Universidad Pedagogica y Tecnologica Tunja, Colombia	May 26
Prof. Rocio Suarez	Universidad del Quindío, Armenia Colombia	June 1
Ms. Alejandra Duarte P.	Universidad de Santander, Microbiología Industrial, Bucaramanga, Colombia	June 3
Mr. Carlos Felipe Espinal, Jorge M. Diaz	Consultants – CIRAD-IICA, Colombia	June15

Activity 3.7. List of visitors, (cont´d)

Name	Institution	Date
Drs. Luis F.Forero, Jose Manuel Suso	CEO and Board Member –Induarroz, Colombia	June 23
11 Farmers	La María, Piendamo, Colombia	July 5
Dr. Shoshi Kikuchi	Head of Laboratory of Gene Expresión of NIAS, Japan	July 18
Dr. Orlando Peixoto de Morais	EMBRAPA Arroz, Brazil	August 11 - 12
Dr. José de Almeida Pereira	EMBRAPA Meio-Norte, Brasil	August 11 - 12
Mr. Juan J. Font and Mr. Jorge Fuentes	Representaciones Comerciales, S.A., Guatemala	August 14 - 19
Dr. Gerard F. Barry	Coordinator Goldenrice Network-IRRI, The Philippines	August 16
Dr. Jonathan Crouch	Genetic Resources Program, CIMMYT, México	August 16
Dr. Eduardo J. Graterol	Research Manager, Fundación para la Investigación Agrícola DANAC, Venezuela	August 17
Drs. Juan J. Font and Jorge Fuentes	Representaciones Comerciales S.A., Guatemala	August 18
6 Students	Universidad San Buenaventura, Cali, Colombia	August 18
Ms. Ana Elizabeth Díaz	Programa MIP, CORPOICA, Palmira	August 19
Dr. Phillipe Prior	CIRAD, France	September 6
Dr. Luis F. Rios	J.P. Gestión Financiera Banco Mundial de Bogotá, Colombia	September 16
Ms. Angela Maria Castaño	Universidad de Caldas	September 22
Ms.Catalina Quintero V.	Universidad de Caldas	September 22
Prof. Henry Toro Lopez	Universidad de Caldas	September 22
Prof. Gerardo Martinez	Universidad de Caldas	September 22
Prof. Luis F. Aristizabal	Universidad de Caldas	September 22
18 Technicians	ICA, Palmira	September 29
Mr. Luis Paulino Herrera and Mr. Mario Jimenez H.	Hortifruti, Costa Rica	September 30
Mr. Luis Paullino Herrera B.	HORTIFRUTI, Costa Rica	October 3 - 8
Mr. Mario Jiménez Hernández	HORTIFRUTI, Costa Rica	October 3 - 8
Dr. Ronald R. Walcott	Associate Professor, Plant Pathology, University of Georgia, USA	October 5 - 8
Dr. Eduardo Villota	President ACOSEMILLAS, Minister Advisor and Board Member of CORPOICA, Colombia	October 13
Dr. Arturo Vega	Executive Director CORPOICA, Colombia	October19
Dr. Kazuhiro Suenaga	International Research Coordinator, JIRCAS, Japan	November 3 - 5
Dr. Shuichi Oshio	Director, Animal Production and grassland Division, JIRCAS, Japan	November 3 - 5

Activity 3.7. List of visitors, (cont´d)

Name	Institution	Date
Dr. Hiroshi Kudo	JIRCAS Representative for South America, Japan	November 3 - 5
Dr. David A. Miron	President, TDM Consultants, USA	November 7
Dr. Nobuyoshi Maeno	Former Member of CIAT – Board Trustees	November 7
Dr. Merion Margaret Liebenberg	Plant Pathologist ARC-Grain Crops Institute, Potchefstroom, South Africa	November 10-16
Dr. Felix Fellmann	Director Asociado, SDC –COSUDE – Central America	November 20-27
Dr. Pedro E. Ferreira Rossi	Director General, CATIE – Costa Rica	November 21-23
Dr. John Beer	Director of Agriculture and Agroforestry, CATIE, Costa Rica	November 21-23
Ms. Isabel Bortagaray	UNU/INTECH, Holland	November 29
Mr. Maryke Labouschange	University of Freestate, Blomfontein	November 30
Dr. Silvia Restrepo	Universidad de los Andes, Colombia	December 5
Dr. Adriana Bernal	Universidad de los Andes, Colombia	December 6
Mr. Robert Kalyebara	Banana Research Program, Uganda	December 8
Dr. Jenny Gaona	SAC, Colombia	December 12
Dr. Mike Meadows	Plant Pest Survey Coordinator- Florida Dpt of Agriculture	December 22

CIAT – Africa

Name	Institution	Date
Ms. Kwesiga Vivian	Trainer, KADFA-ISAMI	March 17
Mr. Ntacyabyikuye Evaristus	Farmer from Rwanda	April 18
Mr. Andrew Guita	CARE, Uganda	April 18 August 5
Dr. Jim Myers	CRSP and Sokoine University of Agric.	April 28
Dr. Phil Miklas	CRSP and Sokoine University of Agric.	April 28
Dr. Susan Nchimbi	CRSP and Sokoine University of Agric.	April 28
Ethiopia NARS	EARO	April 30
Mr. Julius Masanyu	AFRICARE	March - May
Mr. Samwel Ahabwe	NAADS Service Provider	May 21
Mr. Mike Davison	WREN Media	May 25
Mr. Nyiti	FIPS, SARI	August 26
Mr. Mugerwa	Kibo Seed	August 26
Mr. Mushobozi	FLORESTA	August 26
Ms. Edith Banzi	CPP, Armyworm Project, Minjingu Mining Co.	August 26
Prof. Zadoc Ugutu	VicPac, VicRes and Kenya National University	August 18-19
Prof. Kenneth Mavuti	VicPac, VicRes and Kenya National University	August 18-19

Activity 3.7. List of visitors, (cont´d)

Name	Institution	Date
Prof. Mabel Imbuga	VicPac, VicRes and Kenya National University	August 18-19
Mr. Julius Ecuru	VicPac, VicRes and Kenya National University	August 18-19
Dr. Rose Kingamkono	VicPac, VicRes and Kenya National University	August 18-19
Farmers from Kabale	Kabale District	November 1
Mr. Daniel Fotio	IRAD, Cameroon	November 4
Mr. Paul Koona	IRAD, Cameroon	November 4
Mr. Martin Ngueguim	IRAD, Cameroon	November 4
Tarime Farmers in Tanzania	Tarime District	November 23-24

3.8. List of awards to staff in Project PE-1

1) A. Carabalí, A.C. Bellotti and J. Montoya-Lerma. "Honorable Mention" (third place) for the "Hernán Alcaráz Viecco Prize". *Comparing the adaptability of biotype B of* Bemisia tabaci *(Homoptera: Aleyrodiade) to commercial cassava (*Manihot esculenta *Crantz) Mcol 2063 and to wild* M. carthaginensis*.* XXXII Congress of the Colombian Society of Entomology (SOCOLEN), July 2005.

2) E. Álvarez., M. Fregene and X. Hurtado. Honorable mention "Rafael Obregón National Prize of Phytopathology" *"Detección de Marcadores Microsatélites Asociados con la Resistencia al Añublo Bacterial de la Yuca en Colombia"*. July 2005.

3) E. L. Melo., C.A. Ortega., A. Susurluk., A. Gaigl, R. Ehlers and A.C. Bellotti. "Honorable Mention" (third place) "Luis Hernando Pino Santiago Prize", *Search for native populations of entomopathogenic nematodes in regions of Colombia and Panama.* XXXII Congress of the Colombian Society of Entomology (SOCOLEN), July 2005.

4) J.M. Bueno., C. Cardona and P.Chacón de Ulloa. "Hernán Alcaráz Viecco National Prize of Entomology". *Phenology, spatial distribution and development of sampling methods for* Trialeurodes vaporariorum *(Westwood) (Homoptera: Aleyrodidae) on snap beans and beans (*Phaseolus vulgaris *L.)*. XXXII Congress of the Colombian Society of Entomology (SOCOLEN), July 2005.

5) P. A. Sotelo., C. Cardona., G. Sotelo and J. Montoya-Lerma. "Francisco Luis Gallego Prize'' (Second place). Undergraduate thesis presented to the biology academic program, Faculty of Sciences, Universidad del Valle, in the area of forage entomology. *Resistance of* Brachiaria *spp. to the spittlebug: sub-lethal effects of resistant cultivars on adult* Aeneolamia varia *(F.) (Homoptera: Cercopidae)*. XXXII Congress of the Colombian Society of Entomology (SOCOLEN), July 2005.

6) Francisco Morales received the "Distinguished Latin American Plant Pathologist Award" at the XIII Phytopathology Congress in Cordoba, Argentina.

Activity 3.9. Funded special projects developed and executed with partners.

Project Title	Donor	Participating Institutions	Contact Scientist		Total Project budget US$
			CIAT	Participating Institution	
Agricultura de Precisión y la Construcción de Modelos de Campo Cultivo para Especies de Frutas Tropicales (2005 – 2007)	COLCIENCIAS Agencia Colombiana de Cooperación Internacional (ACCI) MADR Colombia	Corporación BIOTEC	Elizabeth Alvarez	Myriam Sánchez	15,280
Assessing the Impact of Biotechnology on Diversity: Effect of Transgenic Maize on Non-Target Soil Organisms. (2002 – 2005)	United States Agency for International Development (USAID)	Cornell University	Anthony Bellotti Jairo Rodríguez	Daniel Peck	99,360.00
Association of Phytoplasma with Cassava Frog Skin Disease (2003 – 2005)	Ministerio de Agricultura y Desarrollo Rural de Colombia (MADR), Colombia	Agrovelez, Jamundí, Colombia Univalle, Colombia	Elizabeth Alvarez	Henry Hamman Diego López	38,720
Desarrollo e Implementación de una Red Nacional para la Certificación Fitosanitaria de Cítricos Competente en el Mercado Mundial (2005 – 2007)	Ministry of Agriculture and Rural Development (MADR), Colombia	CORPOICA, ICA , Profrutales Ltda.	Lee Calvert	Jorge Gómez Jorge E. Angel	261,114
Desarrollo y Tranferencia de Medidas del Manejo del Moko (*Ralstonia solanacearum*), en Cultivos de Plátano del Municipio de Armenia (2005)	Alcadía de Armenia, Colombia	ICA	Elizabeth Alvarez	Cristina Aristizábal	6,000.00

Activity 3.9. Funded projects, (cont´d)

Project Title	Donor	Participating Institutions	Contact Scientist		Total Project budget US$
			CIAT	Participating Institution	
Detecting and Handling Microorganisms (2004 – 2005)	Ministry of Agriculture and Rural Development (MADR), Colombia		Lee Calvert		8,005.00
Dinámica de Fuentes de Inóculo y Análisis de la Estructura de las Poblaciones de los Agentes Causales de Antracnosis en Especies de Frutales Promisorios (2005 – 2007)	COLCIENCIAS, Colombia	CORPOICA	Segenet Kelemu	Jairo Osorio	219,046.00
Evaluación de Aislamientos Colombianos del Virus de la Tristeza de los Cítricos (2003 – 2005)	COLCIENCIAS, Colombia	CORPOICA	Lee Calvert	Jorge Gómez	32,258.00
Evaluación de la Protección Cruzada como Estrategia de Control del Virus de la Tristeza de los Cítricos (2005 – 2007)	COLCIENCIAS, Colombia	CORPOICA	Lee Calvert	Jorge Gómez	35,500.00
Evaluation of the Effectiveness of Biorationals Used in the Management of Bruchid Pests on Beans (*Phaseolus vulgaris*) by Small-Scale Farmers in the Lake Victoria Basin (2004 – 2007)	SIDA SAREC, Sweden	NARS Universities and research programs in Kenya and Tanzania	Eliaineny M. Minja	Mabel Imbuga Paul Tarus Absolom Munyasi John Ogecha Phanice Namungu Hashim Barongo Goodluck Kimaro	30,000.00

Activity 3.9. Funded projects, (cont´d)

Project Title	Donor	Participating Institutions	Contact Scientist		Total Project budget US$
			CIAT	Participating Institution	
Increasing Cassava Productivity through Integrated Pest Management (2005 – 2007)	Inter-American Institute for Cooperation on Agriculture (IICA), Colombia	Live Systems Technology (LST), S.A. , Bogotá , Colombia	Andreas Gaigl	Esperanza Morales	83, 246.00
Integrated Control of Subterranean Pests in South America (2002 – 2005)	BMZ, Germany	University Hannover University Kiel BBA CORPOICA Univ. del Valle Univ. Nacional de Bogotá Univ. de Caldas	Andreas Gaigl	Christian Borgemeister Ralf-Udo Ehlers Gisbert Zimmerman Martha Londoño Miguel Serrano Alberto Soto Luis F. Vallejo	714,961.00
Lulo con Valor Agregado: Nuevas Alternativas para el pequeño Agricultor (2006 – 2008).	Ministry of Agriculture and Rural Development (MADR), Colombia	CORPOICA La Selva, Universidad de Antioquia	Alonso Gonzalez, Zaida Lentini, Elizabeth Alvarez	Mario Lobo	240,513
Manejo Integrado de Enfermedades del Cultivo de Yuca (2005 – 2007)	Ministerio de Agricultura y Desarrollo Rural de Colombia (MADR) and Inter-American Institute for Cooperation on Agriculture (IICA), Colombia	Live Systems Technology (LST) S.A., Colombia	Elizabeth Alvarez	Esperanza Morales Jaime Jaramillo	275,099

Activity 3.9. Funded projects, (cont´d)

Project Title	Donor	Participating Institutions	Contact Scientist		Total Project budget US$
			CIAT	Participating Institution	
Manejo Integrado de la Enfermedad del Moko en Plátano (2005)	CGIAR Award	ICA, CORPOICA	Elizabeth Alvarez	Silverio Gonzalez	10.000.00
Manejo Integrado de la Mosca Blanca en el Trópico – Fase III (2005 – 2008)	Department for International Development (DFID), UK	IITA AVRDC CIP CABI NRI	Francisco Morales	James Legg Peter Hanson Isabel Carballal	2,613,071.00
Mejoramiento del manejo nutricional para el control preventivo del mildeo velloso del rosal *Peronospora sparsa* (2006 – 2007)	COLCIENCIAS, Colombia	CENIFLORES ASOCOLFLORES	Elizabeth Alvarez	Rebeca Lee	104,000.00
Pest and Disease Resistance, Drought Tolerance and Increased Shelf Life Genes from Wild Relatives of Cassava and the Development of Low-cost Technologies to Pyramid them into Elite Progenitors (2005 – 2007)	The Generation Challenge Programme, CGIAR	EMBRAPA-CNPMF, Brazil Namulonge Agricultural and Animal Production Research Institute (NAARI) Crop Research Institute (CRI) National Root Crop Research Institute (NRCRI)	Elizabeth Alvarez, Alfredo Alves Anthony Bellotti Hernan Ceballos Martin Fregene	Anton Bua Titus Alicai Elizabeth Okai Chiedozie Egesi	894,906.00

Activity 3.9. Funded projects, (cont´d)

Project Title	Donor	Participating Institutions	Contact Scientist		Total Project budget US$
			CIAT	Participating Institution	
Promotion of Integrated Pest Management Strategies for Major Insect Pests of Phaseolus Beans in Hillsides Systems in Eastern and Southern Africa (2005 – 2006)	DFID, United Kingdom	NARS in Uganda, Kenya, Tanzania and Malawi	Eliaineny M. Minja Robin Buruchara Kwasi Ampofo	Michael Ugen Fina Opio John Ogecha Felister Makini Catherine Madata David Kabungo Patrick Mviha Barbara Chibambo	113,118.00
Protocol for Monitoring and Developing Resistance to the Bollgard Technology in Colombia (2004 – 2005)	COACOL, Colombia		Anthony Bellotti		30,960.00
Reducción del uso y desarrollo de resistencia a plaguicidas en el cultivo de arroz y fríjol en Colombia, Venezuela y Ecuador (2005 – 2008)	FONTAGRO	INIA, Venezuela FEDEARROZ, Colombia INIAP, Ecuador	Fernando Correa César Cardona	Reinaldo Cardona Miguel Diago Sandra Garcés	224,000 total approved funds 508,000 (with counterpart funding)
Understanding the Mechanism of Plant Resistance to Whiteflies (2004 – 2008)	United States Department of Agriculture (USDA)	USDA	Anthony Bellotti	Stephen Lapointe	61,146.00
Estudios de epidemología y control no convencional de la antracnosis del mango (2006-2009)	COLCIENCIAS	CORPOICA	Segenet Kelemu	Jairo Osorio	70,250

Activity 3.10. List of project proposals and concept notes developed with partners

Donor/Title	Lead Researcher/ Principal Contact	Total Project budget US$
Austria Integrated Soil Fertility, Pest, and Disease Management in the Tropics of America	A. Gaigl	10,000
Austria Improving fruit and vegetable product quality from smallholder system: Optimizing soil-crop-pest management for economically viable, socially acceptable and ecologically sustainable production	A. González T. Oberthur M. Lundy S. Kelemu I. Rao	599,232
Bayer – Dow Dupont - Desarrollo de resistencia a fungicidas en los patógenos del arroz, *Pyricularia grisea* y *Rhizoctonia solani*	F. Correa	30,000
Bayer Cropscience Enhancement of biological control agents through the combination with imidacloprid	A. Gaigl	85,725
Belgium Improving rural livelihoods in Rwanda: Promoting integrated crop, disease, and pest management (ICDPM) strategies for intensification and diversification of agricultural systems.	G.Mahuku R.Buruchara S. Kelemu L. Calvert	5,992,330
BMZ White grubs – soil pests or recycler of soil organic matter. Studies on feeding behaviour of white grubs associated with tuber crops	A. Gaigl	30,000
BMZ Host plant resistance to white grubs attacks	A. Gaigl	30,000
CFC Doubly green beans: Income generation and improved livelihoods for smallholder farmers through an environmentally clean product for local and international markets.	C. Cardona G. Mahuku S. Beebe	2,000,000
CIDA – CIRAD Mejoramiento de la Calidad de Vida de Agricultores bajo Riesgo: Tecnologías y Políticas para Rehabilitar Tierras Degradadas en Cultivos y Pastos en Nicaragua.	C. Lascano I. Rao S. Kelemu J. Peters J.I. Sanz	8,491,127
CIRAD Innovative technologies to reduce diseases in plantain and banana	E. Alvarez	677,000
Colombia – Cuba Desarrollo de prácticas de manejo de pudrición de raíz de yuca mediante la detección molecular de Phytophthora en zonas semi-áridas en Cuba y Colombia.	E. Alvarez	10,927
CYTED Tecnologías innovativas para reducir agroquímicos en plátano y banano	E. Alvarez	248,406
CYTED Implementación de un Sistema Fitosanitario Regional para Centroamérica y el Caribe	L. Calvert	123,000
FONTAGRO Alianzas estratégicas para producción sostenible de yuca en los Andes (with regional partners).	E. Alvarez	300,000
FONTAGRO Tecnologías innovativas para reducir agroquímicos en plátano y banano (with various regional partners).	E. Alvarez	300,000
Ford Foundation Enhance life quality of rural families through the use of sustainable technologies for cassava and plantain.	A Gaigl	308,988

Activity 3.10. List of project proposals, (cont´d)

Donor/Title	Lead Researcher/ Principal Contact	Total Project budget US$
IFAD Development of strategies for integrated soil fertility, pest and disease management on cassava in Colombia, Ecuador, Venezuela and Cuba	A.Gaigl	1,502,208
IPM CRSP Integrated soil pest management for environmentally sound agriculture in South America	A. Gaigl	15,000
Kellogg's Enhance life quality of rural families through training communities in the use of sustainable technologies	A. Gaigl	433,500
MADR Alternativas verdes para el control de enfermedades y plagas en productos de alto valor: Extractos de fique (*Furcraea cabuya*) y swinglia (*Swinglia glutinosa*) como fuentes de sustancias bioplaguicidas naturales. Entidades que presentan la propuesta: CIAT y Universidad del Valle	G. Mahuku C. Cardona	78,671
MADR Alternativas verdes para el manejo sostenible de plagas y enfermedades en cultivos hortícolas de alto valor en Colombia (with CORPOICA, CIPASLA SANOPLANT and CIAT-soils group).	C. Cardona S. Kelemu G. Mahuku	305,944
MADR Estudios de epidemiología y control no convencional de la antracnosis del mango. (CORPOICA and CIAT; CORPOICA is the submitting institution, Approved January 2006)	J. Osorio S. Kelemu	70,250
MADR Mejoramiento de la productividad, la calidad y la inocuidad del cultivo de lulo a través del desarrollo y la implementación de programas de manejo integrado del cultivo y el cumplimiento de buenas prácticas agrícolas internacionales. (Live Science Systems is the submitting institution)	A. Gaigl	218,531
MADR Mejoramiento de la productividad, la calidad y la inocuidad del cultivo de mora a través del desarrollo y la implementación de programas de manejo integrado del cultivo y el cumplimiento de buenas prácticas agrícolas internacionales. (Live Science Systems is the submitting institution)	A. Gaigl	218,531
MADR Mejoramiento de la productividad, la calidad y la inocuidad del cultivo de papa criolla a través del desarrollo y la implementación de programas de manejo integrado del cultivo y el cumplimiento de buenas prácticas agrícolas internacionales. (Live Science Systems is the submitting institution)	A. Gaigl	218,531
MADR Reducción del uso de plaguicidas para aumentar la competitividad de frutas para exportación (with CORPOICA, CIPASLA, Ecoflora, Profrutales and CIAT-soils group).	C. Cardona S. Kelemu	349,650
MADR - IICA Lulo con Valor Agregado: Nuevas Alternativas para el Pequeño Agricultor.	A. González Z. Lentini E. Alvarez	240,513

Activity 3.10. List of project proposals, (cont´d)

Donor/Title	Lead Researcher/ Principal Contact	Total Project budget US$
MADR – IICA Colección, caracterización y multiplicación clonal de selecciones criollas de aguacate con énfasis en la identificación de patrones con tolerancia a *Phytophthora* spp. (Corporación Colombiana de Investigación Agropecuaria-Corpoica, Vivero Profrutales Ltda., Centro Internacional de Agricultura Tropical - CIAT)	E. Alvarez	1,015,012
NZAID Enhance life quality of rural families through the use of sustainable technologies	A. Gaigl	300,000
Palmar del Oriente - Palmas de Casanare - Palmeras Santana - COLCIENCIAS Confirmación de fitoplasma, como agente causante de la marchitez letal en palma de aceite.	E. Alvarez	72,288
Rockefeller Integrated soil fertility, pest and disease management in South America.	A. Gaigl	1,500,0000
USAID Desarrollo de un biofungicida para el manejo de enfermedades de cultivos establecidos en fincas productoras de plátano en Colombia.	E. Alvarez	250,000
USAID Opportunities for the Biologically-base Management of Subterranean Insect Pests (with Cornell)	A. Gaigl	14,000
Not yet submitted to donors		
Biological indicators: Assessing and Monitoring Soil Health using Belowground Indices as Biological indicators. Duration: 5 years	G. Mahuku S. Kelemu	1,500,000
Highlighting available scientific data to address concerns related to modern biotechnology in the East African context.	S. Kelemu L. Calvert	500,000
SSA-CP Improving the resilience of crop/livestock farming system to enhance food security and income generation in SADC countries	G. Mahuku and Others	1,198,465
MCKNIGHT FOUNDATION Promotion of integrated pest and soil management strategies for *Phaseolus* beans in smallholder farming systems in Malawi, Mozambique and Tanzania	Eli Minja	300,000
EU-ASARECA Scaling-out integrated soil and pest management bean based technologies with farmers, Competitive Funding	Eli Minja	351,350
Proposals at initial concept stage		
Biological Pesticides: Exploiting the natural microbial and plant biodiversity wealth for combating diseases and pests for sustainable livelihoods in the tropics. Duration: 5 years	S.Kelemu, C. Cardona	15,000,000
Biocidal proteins from native African plants and microbes for control of plant diseases and pests of major economic importance. (Duration and budget to be defined)	S. Kelemu	
Endophytic fungi and bacteria in the tropics (Africa and Latin America): food safety and crop improvement applications. (Duration and budget to be defined)	S. Kelemu	
Soil biota (System wide Program-IPM)	PE-1 scientists and CGIAR	10,000,000

Activity 3. 11. List of Publications

Refereed journal articles

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Output 4: Global IPM networks (Integrated Whitefly Management Technology) and knowledge systems developed.

Introduction

Phase III of the Tropical Whitefly IPM Project (TWFP) was approved on February 9th, 2005, following modifications made to the original Concept Note submitted to DFID in September 2004. The revised Concept Note included new partners from NRI and new activities, which required a re-organization of the TWFP and its activities, as well as a re-distribution of funds. The modifications introduced responded to:

1. The need to expand activities to the original number of target countries surveyed during Phase I, and include some new countries recently affected by whitefly pests and whitefly-transmitted viruses.

2. The need to maintain the basic composition of the team and research activities pursued since Phase I, in view of the termination of two satellite subprojects of the TWFP in 2004, financed by NZAID (Host Plant Resistance/CIAT) and ACIAR (Begomoviruses affecting tomato in S.E. Asia/AVRDC).

3. The opportunity to bring into the TWFP another international centre (CIP) that had participated in Phase I both with the TWFP (sub-Saharan Africa sub-project) and one of the Crop Protection Programme (CPP) projects (R6617) also working on sweetpotato viruses in SSA.

4. The opportunity to secure the participation of the Farmer Participatory Research (FPR) Working Group (led by CABI, UK), and the Impact Assessment Working Group (led by CIP) of the Systemwide IPM Programme.

5. The proposal advanced by NRI and NRInternational to assure the continuity (beyond the requested one year extension) of two of the CPP projects (R6617 and R6627(working in India (whitefly-borne tomato viruses) and SSA (sweetpotato viruses), that had previously collaborated with the TWFP since Phase I.

To this end, a Planning Meeting was organised by the Coordinator of the TWFP, with the collaboration of Dr. Frances Kimmins of NRI, in London, on 28 February and 1 March, 2005. The participants in this Task Force were: Frances Kimmins, NRI; Tim Chancellor, NRI; Richard Gibson, NRI; John Colvin, NRI; Barbara Adolph, NRI; James Legg, IITA/NRI; Pamela K. Anderson (CIP); Peter Hanson (AVRDC); Janny Vos, CABI-UK; Anthony Bellotti, CIAT; Cesar Cardona, CIAT; and Francisco J. Morales, CIAT. The primary objective of this meeting was to discuss future collaborative activities, and to produce a final Project Framework for Phase III.

This working group defined goal and objectives of Phase III as follows:

Goal: To promote sustainable agriculture and socio-economic growth in resource-poor farming communities possessing mixed cropping systems affected by whitefly pests and whitefly-transmitted viruses in Sub-Saharan Africa (SSA), southern Asia and tropical Latin America.

Objectives

1. To provide reliable information and technical assistance to small- (< 3 ha) and medium-scale (3-8 ha) farmers, on the biology, dissemination, and integrated management of whiteflies and whitefly-transmitted viruses affecting major food and cash crops in the Tropics.

2. To instruct farmers about the diverse negative consequences of misusing insecticides to control whiteflies, emphasizing the need to reduce yield losses, production costs, environmental and food contamination, human health risks, and the gradual development of resistance to insecticides in whitefly pests.

3. To establish sustainable mixed cropping systems in order to promote food security and economic growth in small- and medium-scale farming communities seeking to diversify their traditional food staples with high-value horticultural crops.

Achievements

All sub-projects have initiated activities according to the Logframe and proposed Workplan. The major achievement for Phase III has been the tangible results obtained by the TWFP and related CPP projects in the previous two phases. For instance, sub-project 1 has been extremely successful in diagnosing the nature of the severe outbreaks of Cassava Mosaic Disease (CMD) in SSAfrica, and, more important, in containing these outbreaks by deploying CMD-resistant germplasm. This experience and the complementary IPM measures validated, will be the base for the successful dissemination of this work throughout SSAfrica. The work on sweet potato in this region is regarded as a very complementary effort to safeguard food security in SSA and improve the nutritional value of sweet potato, by linking these activities with the promotion CIP is doing of its orange-fleshed (VitA-rich) sweet potato germplasm.

In Central America, the new begomovirus-resistant common bean cultivar EAP-951077, has been accepted as a superior variety by 92% of 60 farmers selected in Phase II to multiply the seed. Phase III will see a significant increase of the area planted with this material in Central America. During this semester, farmers in the Valley of Zapotitan, El Salvador (Pilot Site of the TWFP) harvested over 60 tons of seed to distribute in the country. Similar activities are now reported from Honduras and Nicaragua. The use of physical barriers to protect horticultural crops against whitefly- and other insect-borne viruses, has been promoted by different projects in Central America and Mexico. In Colombia, this strategy is also being promoted at present. Some farmers claim that the adoption of this technique reduces the cost in pesticide inputs by 60%, and triples the yield of uncovered controls.

The profit made by tomato farmers in India has been increased up to 10 times following the adoption of the virus-resistant tomato varieties promoted by the CPP project led by Dr. John Colvin with the collaboration of AVRDC and the University of Agricultural Sciences of Bangalore. The promotion of these varieties has continued this semester as a joint effort of the CPP and TWFP. More virus-resistant materials are currently being selected by AVRDC for India and Latin America.

The Andean subproject has successfully tested and validated the IPM technologies based on the identification of an ‘action threshold’, at which point, farmers have to control the population of *Trialeurodes vaporariorum*. This recommendation drastically reduces unnecessary insecticide applications on a preventive basis, the leading cause of pesticide abuse. This subproject has produced substantial data on the socioeconomic situation of small-scale farmers affected by the whitefly *T. vaporariorum*, and the benefits accrued from the adoption of IPM strategies. These data and

conclusions will be published shortly, and the experience accumulated will be transferred to the Bolivian highlands.

The search for host plant resistance in cassava against whitefly pests, continues to yield positive results both against the main whitefly pest in South America, Aleurotrachelus socialis, and B. tabaci in Africa. Current efforts are directed towards increasing the resistance to B. tabaci in CMD-resistant cassava cultivars.

Activity 4.1. Monitoring of whitefly populations in the Andean zone

Contributors: I. Rodríguez, C. Cardona, and H. Morales

Highlight:

- Detected important changes in whitefly species composition in the target area

Rationale

Continuous monitoring of changes in whitefly populations and species composition in target areas is one of the most important objectives of the DFID-funded project on Sustainable Management of Whiteflies. This is needed to develop appropriate management systems and, if necessary, to modify existing systems so as to be able to cope with new situations.

Materials and Methods

In 2005 we processed a total of 53 whitefly samples (adults and pupae) collected in 20 locations of the Cauca Valley region of Colombia, at altitudes ranging from 950 to 2200 meters. Samples were taken from beans, snap beans, squash, pepper, cucumber, tomatoes, melons, and several other annual crops. When possible, identification was initially based on morphological characteristics of pupae. To differentiate between biotypes (which are impossible to differentiate by morphology), we used RAPD techniques (primer OPA-04). RAPD patterns of pupae and adults collected in the field were compared with those of existing mass rearings of different whiteflies maintained at CIAT (Figure 4.1.1).

Results and Discussion

Analysis of the 53 samples taken in the Cauca Valley (Colombia) showed that 43.4% of the whiteflies collected belonged to the B biotype of *Bemisia tabaci*, the most aggressive form of whitefly known to date. The B biotype was found affecting pepper, cucumber, tomato, melon, snap beans and several other vegetables, as well as soybeans, cotton, and other crops. As shown in Figure 4.1.2, species composition in the Cauca Valley has changed drastically in the past eight years and the trend continues: since its introduction to Colombia (Quintero *et al*., 1998, Rev. Col. Entomol. 24: 23-28.) the B biotype is occupying niches previously reserved to the A biotype or to *T. vaporariorum* even in areas located above 1000 meters. *T. vaporariorum,* the predominant species in 1997 is now the second most important species (24.5% of samples) in areas located below 1000 meters above sea level. In many locations, *T. vaporariorum* is associated with *B. tabaci* (biotypes A and B). The B biotype is an aggressive form of whitefly that is causing all the serious problems described in our 2003 and 2004 Reports. These include physiological disorders in several different crops (pod chlorosis in snap beans, silvering of leaves in squash, uneven ripening of tomatoes), and the ability to transmit a geminivirus that has devastated snap beans in areas below 1200 meters.

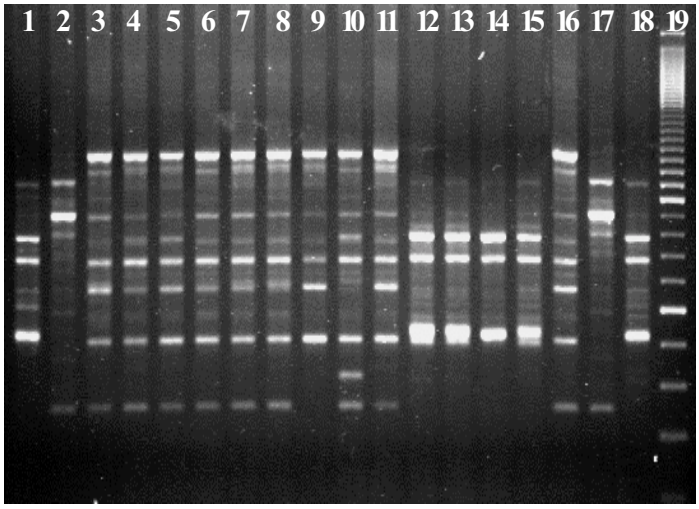


Figure 4.1.1. RAPD's for whitefly adults and pupae collected in the Cauca Valley region of Colombia. Amplification using the OPA-04 primer: 1, 2, 3, *T. vaporariorum, B. tabaci* biotype A, and *B. tabaci* biotype B adults, respectively, from reference rearings maintained at CIAT; 4-5, *B. tabaci* biotype B adults collected on squash in Quintero, Roldanillo (934 masl); 6-7, *B. tabaci* biotype B pupae collected on squash in Turin, Candelaria (999 masl); 8-9, *B. tabaci* biotype B adults collected on snap beans in Villagorgona, Candelaria (998 masl); 10-11, *B. tabaci* biotype B pupae collected on tomato in La Regina, Candelaria (1015 masl); 12-13, *T. vaporariorum* adults collected on snap beans in El Pedregal, Florida (1109 masl); 14-15, *T. vaporariorum* pupae collected on snap beans in El Pedregal, Florida (1096 masl); 16, 17, 18, *B. tabaci* biotype B, *B. tabaci* biotype A and *T. vaporariorum* adults, respectively, from reference rearings maintained at CIAT; 19, Molecular marker (100 pb).

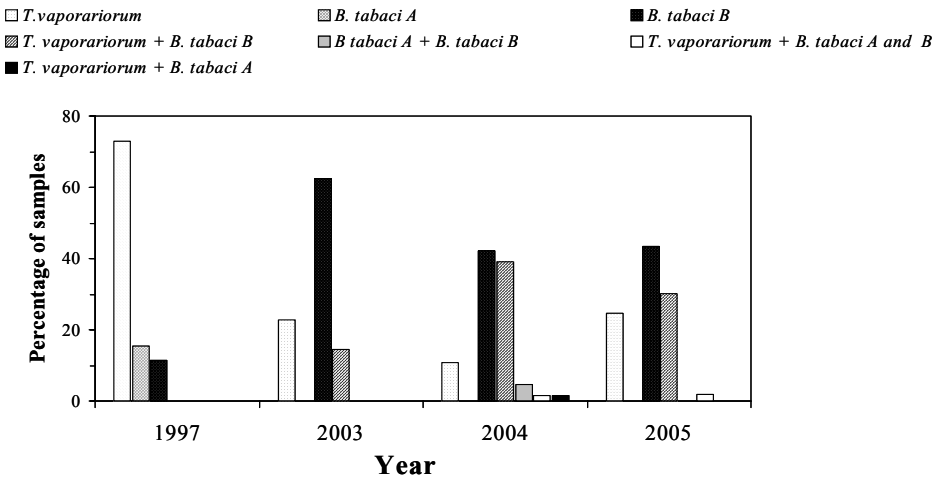


Figure 4.1.2. Changes in whitefly species composition in the Cauca Valley region of Colombia (1997-2005).

Activity 4.2. Monitoring of insecticide resistance in whitefly populations

Contributors: I. Rodríguez, H. Morales, and C. Cardona

Highlight:

- Detected varying levels of resistance or susceptibility to some of the insecticides commonly used for whitefly control in the target region

Rationale

Monitoring of insecticide resistance is another major objective of the DFID-funded project on Management of Whiteflies in the Tropics. Both major whitefly species and their biotypes in the Andean zone are the targets of excessive use of insecticides. This is reflected in ever increasing levels of resistance to insecticides (Cardona *et al*., 2001, Rev. Col. Ent 27: 33-38.) and difficulties in control. The main purpose of a continuous monitoring of insecticide resistance is to develop alternative management strategies that will help to overcome resistance or delay the onset of this phenomenon.

Materials and Methods

Using previously established diagnostic dosages for nymphs, and adults, we tested populations of whiteflies in the Cauca Valley in Colombia. Adult resistance levels were monitored under field conditions by means of the insecticide-coated glass vial technique. Resistance of first instar nymphs was measured using the foliage dipping technique. Systemic novel insecticides (mostly neonicotinoids) were tested using the petri dish technique (see 2003 and 2004 PE-1 Annual Reports).

Results and Discussion

No major changes were detected. In general, the response of *T. vaporariorum* adults (Table 4.2.1) and nymphs (Table 4.2.2) is still that of susceptibility to the insecticides that were tested. A reduced response to the neonicotinoids imidacloprid and thiamethoxam and to the insect growth regulators buprofezin and diafenthurion in host spots like Pradera deserves continuous monitoring.

Table 4.2.1. Response (percentage corrected mortality) of adults of *Trialeurodes vaporariorum* to six insecticides in three areas of the Cauca Valley region of Colombia.

Race	Percentage corrected mortality	
	methomyl (2.5ε g/vial)	imidacloprid SC (40 ppm)
'CIAT'[a]	95.3a[b]	91.2a
Tenerife	96.0a	92.4a
El Dovio	95.1a	87.9a
Pradera	88.2b	87.6a
C.V.(%)[c]:	4.7	7.3
	thiamethoxam (200 ppm)	imidacloprid WG (0.5 g/l)
'CIAT'	91.3bc	94.4b
Tenerife	95.0ab	96.0ab
El Dovio	98.5a	98.5 a
Pradera	88.0c	96.3ab
C.V.(%):	5.3	4.3
	thioxyclam hydrogen oxalate (0.5 g/l)	imidacloprid + ϐ cyfluthrin (2.5 cc/l)
'CIAT'	89.3b	98.4a
Tenerife	98.5a	99.0a
El Dovio	92.5b	100.0a
Pradera	98.5a	99.5a
C.V.(%):	5.4	2.3

[a] Susceptible strain maintained at CIAT

[b] For each product, means within a column followed by the same letter are not significantly different at the 5% level by LSD. Each product was analyzed separately

[c] Coefficient of variation.

Table 4.2.2. Response (percentage corrected mortality) of nymphs of *Trialeurodes vaporariorum* to six insecticides in three areas of the Cauca Valley region of Colombia.

Race	Percentage corrected mortality	
	buprofezin 9.2 ppm	diafenthiuron (60.1 ppm)
'CIAT'	100.0a[a]	90.0ab
Tenerife	90.3a	97.4[a]
El Dovio	100.0a	98.9[a]
Pradera	76.1b	77.9b
C.V.(%)	8.7	12.2
	imidacloprid SC (171.5 ppm)	thiamethoxam (0.5 g/l)
'CIAT'	100.0a	100.0a
Tenerife	99.1ab	99.8a
El Dovio	100.0a	99.0a
Pradera	93.6b	78.7b
C.V.(%)	4.0	3.7
	imidacloprid + ƀ cyfluthrin (2.5 cc/l)	imidacloprid WG (0.5 g/l)
'CIAT'	100.0a	100.0a
Tenerife	100.0a	99.3a
El Dovio	100.0a	100.0a
Pradera	94.0b	85.0b
C.V.(%)	2.1	4.7

[a] Susceptible strain maintained at CIAT
[b] For each product, means within a column followed by the same letter are not significantly different at the 5% level by LSD. Each product was analyzed separately
[c] Coefficient of variation.

Activity 4.3. The development of "rapid selection" method to determine whitefly resistance in cassava genotypes.

Contributors: A. Carabali and A. C. Bellotti.

Highlight:

- The ovipositional rate (N_o. eggs/female) of the cassava whitefly *Aleurotrachelus socialis* on a given genotype is a good indication of the level of resistance in that genotype.

Rationale

The cassava whitefly, *A. socialis*, causes direct damage to cassava in the neotropics by feeding on the pholoem of leaves, inducing leaf chlorosis and abscission which can result in considerable reduction in root yield during prolonged attack. Because of cassava's long growth cycle (8 to 24 months), the continued use of chemical pesticides for whitefly control increases production costs and is uneconomical for the small farmer (Holguin and Bellotti, 2004, Revista Colombiana de Entomología

30: 37-42.). Stable host plant resistance (HPR) offers a practical, low cost, long-term solution for maintaining reduced whitefly populations. Whitefly resistance is rare in cultivated crops; however, good sources of resistance to *A. socialis* have been detected in several *M. esculenta* genotypes and high yielding hybrids with moderate levels of whitefly resistance are being developed (Bellotti and Arias, 2001, Crop. Prot. 20: 813-823). In addition, a preliminary evaluation of wild *Manihot species* has revealed high levels of resistance to *Aleurotrachelus socialis* in accessions of *M. flabellifolia* and *M. esculenta* subsp *peruviana*.

The development of cassava hybrids with whitefly resistance will involve screening a considerable number of F1 progeny from crosses between two or more *M. esculenta* genotypes and/or the progeny from interspecific crosses between *M. esculenta* and wild *Manihot* species such as M. *flabellifolia.* Projects to accomplish this goal are already in progress. A mechanism is needed that can speed up the evaluation of a large number of genotypes for whitefly resistance. Previous evaluations of whitefly resistance in *M. esculenta* genotypes had detected a possible correlation between resistance and low oviposition rates (Bellotti and Arias, 2001, Crop. Prot. 20:813-823.). Evaluations of the interrelations between ovipositional preference by females for specific genotypes and the development, survival and reproduction of immature offspring constitutes a crucial point in theoretical host plant/insect relationships (Thompson, 1988, Entomol. Exp. Appl. 47: 3-14).

The objective of this present study is to evaluate ovipositional preference/non-preference in *A. socialis* for specific cassava genotypes as a possible "rapid selection" process for resistant/susceptible genotypes.

Materials and Methods

Ten plants, progenies of an interspecific cross between *M. esculenta* and M. *flabellifolia* (CW 235-72, CW 259-3, CW 259-43, CW 257-10, CW 258-17 and CW 259-10) and the commercial variety CMC-40, were planted in plastic pots at a depth of 15 cm. Five forty day old plants of each genotype were placed in wooden/nylon mesh cages (1 m x 1 m x 1 m) for whitefly infestation. Adult *A. socialis* individuals were harvested from the CIAT established colony being maintained in the greenhouse (25 $\pm$ 5°C, 70 $\pm$ 5% RH and 12 hr photoperiod). Studies on ovipositional preference and nymphal development were carried out in the greenhouse. Ten pair (10 males 10 females) of recently emerged *A. socialis* adults (sexed according to the technique described by Eichelkrant and Cardona, 1989) were harvested from the CIAT established colony on CMC-40. Each adult pair was placed in a small leaf cage (2.5 cm diameter x 2.0 cm depth) and attached to the underside of leaves of each genotype (Figure 4.3.1a). Adults were removed after five days and the number of eggs oviposited were recorded (Figure 4.3.1b). Oviposited eggs were allowed to develop for 10 additional days in order to determine the number that will develop to the third nymphal instar.

Figure 4.3.1. a) A small leaf cage for confining whitefly females in oviposition studies; b) *A. sociales* eggs oviposited on cassava leaf undersurface

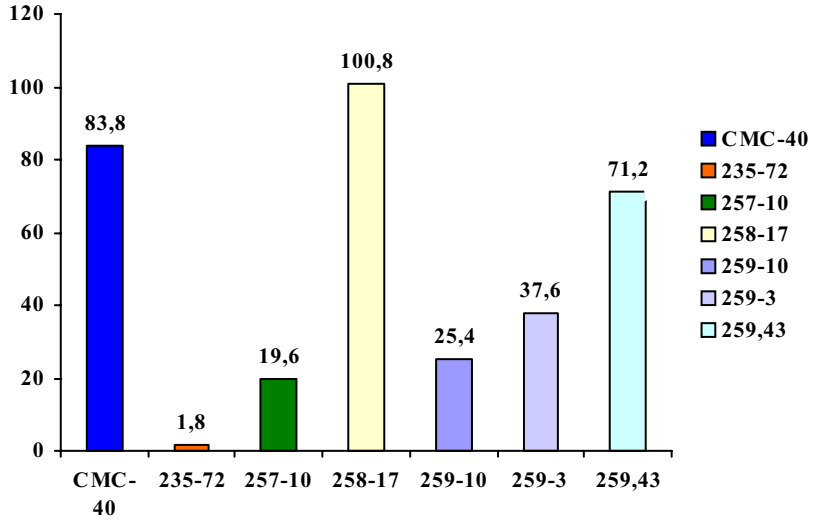


Figure 4.3.2. Genotypes Oviposicional rates (No. eggs/female/5days) of *Aleurotrachelus socialis* on cassava genotypes.

Statistical analysis was carried out using the Stat View, version 5.0.1 (SAS Institute, 1999) program. Differences between average values for oviposition and nymphal development were analyzed using ANOVA. Multiple comparisons were preformed by the Fisher test.

Results and Discussion

Development of *A. socialis* nymphs on cassava (interspecific progeny) genotypes displayed a behavior similar to the rate of oviposition on these genotypes. The genotype CW 235-72 had the lowest ovipositional value (1.8 eggs/10 females) when compared to other genotypes such as CMC-40 (83.8 eggs), CW 258-17 (100.8), CW 259-43 (71.2) (Figure 4.3.2)

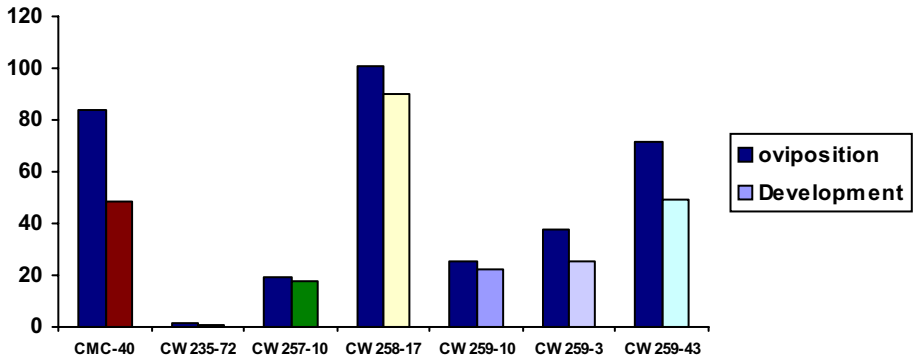


Figure 4.3.3. *Aleurotrachelus socialis* oviposition and nymphal development to the third instar on cassava genotypes

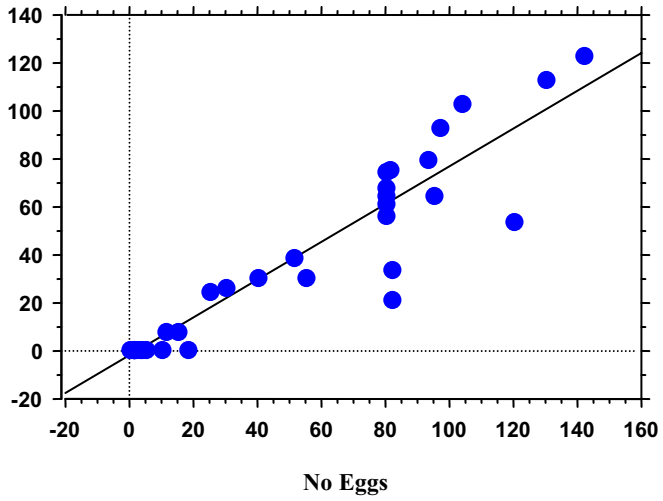
(ANOVA-Fisher's $P < 0.05$). The differences in oviposition expressed in this test is an indication that this variable can be used to distinguished between resistant and susceptible genotypes. This was a no-choice test so the ovipositional rates expressed indicate the ability of *A. socialis* females to oviposit on that genotype.

It is not yet understood why females will oviposit so few eggs on genotypes such as CW 235-72, CW 257-10 (19.6) and CW 259-10 (25.4) when no other choice (genotype) is available. A free-choice test among these genotypes might provide additional information on the mechanism involved in ovipositional preference.

The genotype CW 235-72 had the least development of nymphal stages, owing to the very low ovipositional rates. CMC-40 and CW 259-43 displayed the highest percentage difference (42% and 30% respectively) in nymphal development when compared to the initial ovipositional rates (Fisher's $P < 0.05$). (Figure 4.3.3).

The genotypes CW 235-72 and CW 257-10 showed the least difference (0% and 17% respectively) between oviposition and nymphal development.

A regression analysis was conducted to obtain a correlation between oviposition and nymphal development (3rd. instar) of *A. socialis* on progeny of *M. esculenta* x M. *flabellifolia* (Figure 4.3.4). Results show an 87% correlation in development of the nymphal stages and number of eggs oviposited. It can therefore be concluded that the number of eggs oviposited on a given genotype can be used as an indicator of the preference/resistance of *Aleurotrachelus socialis* for that genotype.



Y = -1.875 + .786 * X; R^2 = .873

Figure 4.3.4. Correlation between oviposition and nymphal survival of the whitefly *Aleurotrachelus socialis* on cassava genotypes (from interspecific crosses of *M. esculenta* x *M. flabellifolia*).

Activity 4.4. Determining the plant metabolites involved in whitefly (*Aleurotrachelus socialis*) resistant cassava varieties, MEcu 64, MEcu 72 and MPer 334

Contributors*:* D, F. Múnera, S. Lapointe, A.Valencia, P. Calatayud and A. C. Bellotti.

Highlights:

- Research on the biochemistry of whitefly resistance in cassava indicates that a relationship may exist between proteins and the presence of resistance to the species *Aleurotrachelus socialis*.

- Research indicates that a relationship may exist between proteins in the cassava leaf and resistance to the whitefly, *Aleurotrachelus socialis*.

Rationale

The whitefly, *Aleurotrachelus socialis*, is a major pest of cassava, reducing root yield and the formation of cassava planting material (cuttings or stakes). Field evaluations during a 1, 6- and 11-month attack resulted in yield losses of 5, 42 and 79% respectively (Bellotti and Vargas, 1986 [Conjunto audiotutorial], CIAT). Whiteflies cause direct damage to cassava by feeding on the phloem of leaves, inducing leaf chlorosis and abscission, which results in reduction in root yield if feeding is prolonged. Additional yield reduction can be caused by the growth of a "sooty-mold" on whitefly exudates deposited on cassava leaves that deters photosynthesis.

The CIAT cassava germplasm bank contains nearly 6000 accessions, of which 93% are landraces (locally selected cultivars), collected from tropical and subtropical regions of the world, but mainly from the Neotropics. This germplasm collection has been extensively screened in the field for whitefly (*A. socialis*) resistance and more than 5400 landrace cultivars have been evaluated. Sources of resistance to *A. socialis* have now been identified. The clone "MEcu 72" has consistently expressed high level of resistance.

Several additional cultivars, including "MEcu 64; MPer 334, MPer 415, MPer 317, MPer216, MPer 221, MPer 266 and MPer 365, have expressed moderate to high levels of resistance. These results also indicate that *A. socialis* resistance may be concentrated in Peruvian and Ecuadorian germplasm. Greenhouse and field studies show that *A. socialis*feeding on resistant clones have less oviposition, longer development period reduced size and higher mortality than those feeding on susceptible ones (Arias, 1995, MSc thesis, Univ. Nacional de Colombia, Palmira, Colombia, pp 164). *A. socialis* nymphal instars feeding on MEcu 72 suffered a 72.5% mortality, mostly in the early instars (Bellotti and Arias, 2001, Crop Protection 20:813-823).

Recent studies under controlled conditions in the growth chamber, *A. socialis* had a longer development cycle when feeding on MEcu 64, MEcu 72 and MPer 334 when compared to the susceptible control, CMC 40. Nymphal mortality was highest on MPer 334 (77.5%), followed by MEcu 64 and MEcu 72 with 68.5% and 68.0%, respectively.

In addition, genomic sequences possibly involved in *A. socialis* resistance have been detected in MEcu 72 using AFLP and microsatelite markers (Bellotti, *et al.,* 2003, Memorias XXX Congreso SOCOLEN, Colombia).

Plant strategies for resisting insect attack often involve biochemical factors or activities. Studies were therefore initiated to determine what plant metabolites might be involved in the development of *A. socialis* resistance found in the resistant genotypes. MEcu 64, MEcu 72 and MPer 334.

Materials and Methods

Whiteflies have piercing-sucking feeding habits; this has made it difficult to develop an artificial liquid diet that would allow testing the biological activity of protein extracts for each of the resistant and susceptible genotypes to determine the relationships between the protein and resistance to the whitefly.

The plan includes obtaining polyclonal antibodies from the immunization of rabbits against protein extracts for each of the materials, and later to determine by means of immunodetection, and the combination of Western Blot and 2D SDS-PAGE techniques, the differences between each of the protein extracts. The resistant genotypes evaluated were MEcu 72, MEcu 64 and MPer 334. The susceptible control was the genotype CMC40. This process will be carried out using healthy plants (non-infested), and plants infested with *A. socialis*, for each of the genotypes, to detect if a proteic response occurs in infested plants. In addition, *A. socialis* feeding on resistant plants will be examined for the presence of a plant protein.

Electrophoresis, employing polyacrylamide gels (PAGE), has proven to be a very useful technique for the analysis and characterization of complex protein mixtures. Nevertheless, since access into the interior of protein matrixes is limited, information generated about the individual components is usually restricted to molecular weight and isoelectric dots. The transfer of proteins by PAGE to an unfixed membrane permits the utilization of diverse tests for an improved characterization. One of the more precise applications for the transfer of proteins to membranes, is through immunodetection which consists of the identification and characterization of a fixed antigen by means of antibody tests (Timmons and Dunbar, 1990, Methods in Enzymology 182: 678-688; Garfin, 1990, Methods in Enzymology 182: 459-477).

Inmune-detection permits estimating by semiquantitative means, the mass or abundance of a specific protein in a determinate tissue. This technique is regularly employed in experimental studies in which the objective is to detect a specified protein or to observe its variation under diverse conditions.

Total Protein Extraction: To extract the total protein, cassava leaves (without petioles) were macerated in liquid nitrogen, obtaining a very fine powder that was subsequently homogenized for five hours at 4ºC with the buffer Tris HCL, pH 8.0, and containing 1mM of EDTA (metalloprotease inhibitor), 5 mM of DTT (reduction agent), 1% PVP (antiphenolic), and 5 mM of PMSF (serine protease inhibitor) at a proportion of 1g macerated leaf to 3ml of buffer. The following step consisted of filtering this mixture and centrifuging it at 15000 rpm for 30 minutes at 4ºC, to clarify the extract and eliminate vegetative tissue. The supernadant is dialyzed with a dialysis membrane of W.M. Co. 3.5 Kd and finally lyophilized to obtain an extract in powder form, in order to manipulate the concentration by weight units.

Immunization and Production of Polyclonal Antibodies against Cassava Proteins: Polyclonal antibodies were used as they contain different sub-classes of antibodies, including IgG, IGM, IGE, IgA and IgD. Each antibody represents the product of only one stimulated lymphocyte and its clonal progeny. An antigen complex such as a protein can contain several distinct or epitopes or determinant antigens, each of which is specifically recognized by antibodies from only one clonal lymphocyte (Dunbar and Schwoebel, 1990, Methods in enzymology 182: 663-670).

To produce polyclonal antibodies the following steps were developed:

- ü Two milligrams of each protein was dissolved in 1 ml of the buffer Tris-Glicina pH 6.8 and later emulsified with one ml of Freund's complete adjuvant.
- ü Four New Zealand breed rabbits were employed. Each of them was subcutaneously injected four times with 0.5 ml of each of the prepared proteins. The injections were applied to the animal's loin.
- ü After three weeks, the four applications were repeated on each rabbit, but at this time the proteins were emulsified with 1ml of Freund's incomplete adjuvant. Two of the injections were intermuscular.
- ü Ten days after the last injections, the animals were bled, obtaining 15-20 ml of blood from each.
- ü The collected blood was left at room temperature for 24 hours, than centrifuged and the serum was stored coagulated in aliquots for later analysis.

Test for Antibody Recognition using the Dot Blot Technique: A test for antibody recognition using the Dot Blot technique was carried out to verify that the antibodies produced were in good condition. The following steps were developed:

- ü One milligram of each of the proteins was dissolved with 200 ε1 of Tris Glycine (pH 6.8) buffer. On each nitrocellulose membrane 5 ε1 of the stock solution was applied to each of the proteins.
- ü Blockage of the nitrocellulose membrane with the sample in TBS containing 1% gelatin.
- ü Exposure of the membrane to 30 ε1 of the first antibody dissolved in 30 ml of blockage solution.
- ü Four washings of the membrane of 15-minutes each. The first three with TTBS (TBS containing 1% tween 20) and the last with TBS.
- ü Exposure of the membrane in 30 ε1 of the second antibody (Bound to PER) dissolved in 30 ml of the blockage solution.
- ü Four washings of the membrane of 15-minutes each. The first three with TTBS (TBS containing 1% tween 20) and the last with TBS.

ü Addition of 5 ml of revealed solution (40 ml of TBS, 3 ε1 of hydrogen peroxide and 30 mg of 4 Chloro-1-Naphtol dissolved in 10 ml of methanol). This solution is preheated at 35°C.

SDS-PAGE Electrophoresis: Using electrophoresis trials with polyacrilamide gels in disnatured conditions (SDS-PAGE) it was determined:

ü Protein sample concentrations (mg/ml) carried on gel pools for a visualization of the bands. To do this, concentrations of 200 mg/ml, 100 mg/ml, 75 mg/ml, 50 mg/ml, 25mg/ml, 10 mg/ml and 2mg/ml were tested.
ü Adequate concentrations of the resolving phase of the gel were achieved for a good view of the protein bands. To do this, concentrations of 10%, 14%, and 17% were tested. It should be noted that the phase stacking concentration was 4% at all times.
ü Polymorphism by molecular weight for each of the proteins for each genotype evaluated. To do this a marker of the Prestained SDS-PAGE from Biorad Laboratories (with an arrange of 106 to 20.8 Kd) molecular weight was utilized.

These tests were carried out in a Biorad Mini Protean electrophoresis chamber and followed the protocol established by the manufacturer for both the electrophoresis as well as the staining of the gels.

First immunization tests were with healthy plants (no whitefly infestation) using the Western Blot Technique. This test seeks to determine the specificity of each antibody in the genotypes being evaluated. An SDS-PAGE electrophoresis of the proteic extracts was carried out for each of the genotypes, using the previously determined conditions. Subsequently a transfer of the bands obtained during electrophoresis was made to a nitrocellulose membrane using the Western Blot Technique. Antibody recognition was determined using the Dot Blot Technique, with the exception of the first two steps.

Results and Discussion

Tests for antibody recognition using Dot Blot: By using the afore-described methodology a clear recognition of the antibodies for each of the genotype extracts was achieved and evaluated. In addition a good staining (concentration) of the polyclonal antibodies originating from each genotype was observed, owing to the high intensity of each marker (Figure 4.4.1).

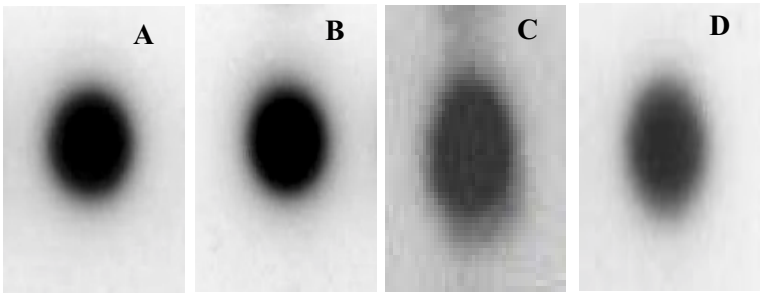


Figure 4.4.1. Test for antibody recognition using the Dot Blot technique. A: antibodies against MEcu 72, B: antibodies against MEcu 64, C: antibodies against MPer 334, D: antibodies against CMC 40.

These results indicate that the process for immunization and production of the antibodies using the described procedures was successful; therefore it is possible to continue with the cross-tests for immunodetection of proteins for both the varieties being evaluated, as well as for *A. socialis*.

SDS-PAGE Electrophoresis: It was determined that the protein sample concentration that best provides a good visualization of the bands is 2mg/ml. This concentration provided for well defined bands without vertical streaking of protein, as occurred with the other concentration evaluated (Figure 4.4.2).

The protein concentration that gave adequate results for the resolving phase by providing good visualization of the protein bands was 14% (Figure 4.4.2). With the other concentrations the distribution of the bands along the gel were not uniform and very congested on the lower part of the get at the 10% concentration, while they were congested at the top of the gel at the 17% concentration.

In Figure 4.4.2, polymorphic bands can be observed between the resistant and susceptible genotypes, with molecular weights between 47.5 and 35 Kd. A common polymorphic band is clearly noted in the resistant genotypes (black arrows), although it is less intense for MPer 334. The genotype MEcu 64 shows a high polymorphism as well as an additional band that is absent in the other genotypes (yellow arrow). The yellow circle on Figure 4.4.2 indicates the absence of these aforementioned protein bands on the susceptible genotype, CMC 40. These results are a good indication that these protein immunodetection tests should be continued on these genotypes; the differences shown between the resistant and susceptible genotypes are a good indication that a relationship may exist between these proteins and the presence of resistance to *A. socialis*.

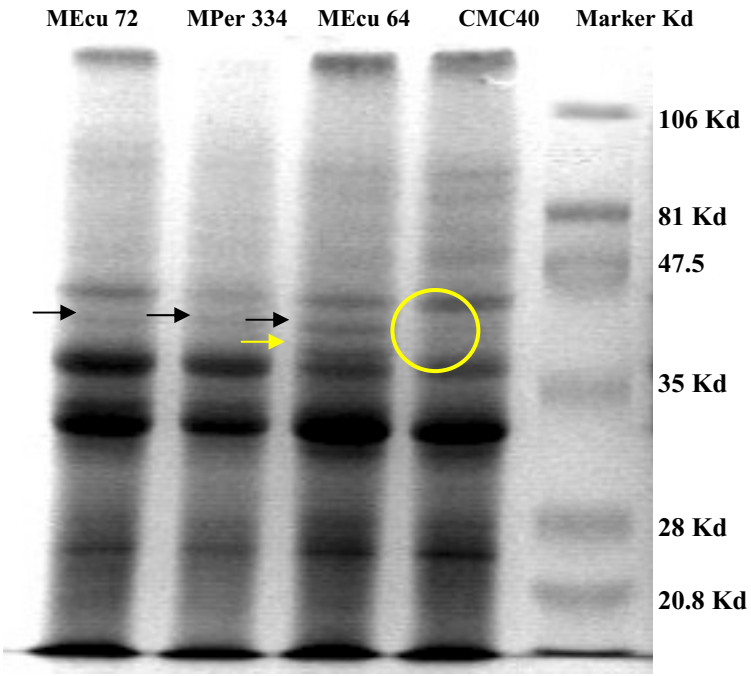


Figure 4.4.2. SDS-Page. Phase resolving concentration of 14%, Sample concentration of 2 mg/ml. The black arrow indicates the polymorphic band commonly present in the resistant genotypes and absent in the susceptible, CMC 40, indicated by the yellow circle. The yellow arrow shows an additional polymorphic band that is only evident in the resistant genotype MEcu 64.

First immunization test with healthy plants (no whitefly infestation) using the Western Blot Technique: Antibody specificity obtained from MEcu 64 with proteins originating from the same genotypes (B pool 3) can be observed in Figure 4.4.3. The four bands located in different positions, as indicated by the arrows, can only be observed in this genotype and are absent in the control (CMC 40) and the other resistant genotypes. These results support those obtained from SDS-PAGE electrophoresis, indicating that this genotype is markedly different than the other genotypes and this could be related to whitefly resistance.

In general, common bands, for all of the genotypes combined with all of the antibodies can be observed. This indicates common proteins, be they structural or functional, in the genotypes.

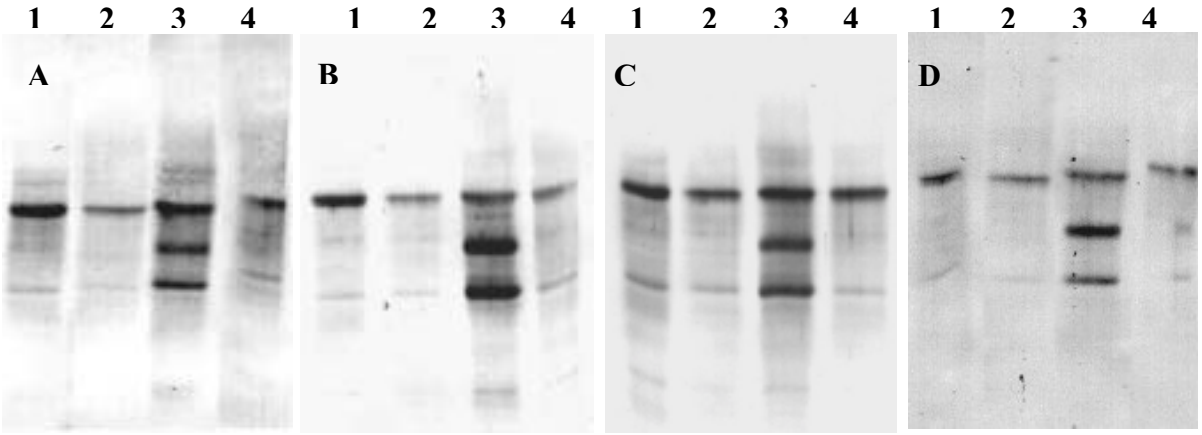


Figure 4.4.3. Immunodetection of healthy genotypes (non whitefly infested) using the Western Blot Technique. 1: MEcu 72, 2: MPer 334, 3: MEcu 64, 4: CMC 40. A) developed with antibodies of CMC 40; B) developed with antibodies from MEcu 64; C) developed with antibodies of MEcu 72; D) developed with antibodies from MPer 334. The black arrows signal the polymorphic bands of MEcu 64 that are absent in the other genotypes.

Projections

Present results indicate a distinct difference in the proteic behavior of at least one of the genotypes when free of whitefly (*A. socialis*) infestation. Therefore the following processes and activities are suggested:

- Development of a well defined proteic profile for each of the genotypes using more sensitive techniques, such as silver staining. This profile would be used as a reference for the protein behavior of each genotype in the absence of whitefly infestation and utilized for future comparisons.
- Obtain a proteic profile for whiteflies feeding on susceptible plants using more sensitive techniques. This profile would also be used as a protein behavior reference for whiteflies feeding on non-resistant genotypes.
- Determine the proteic profiles for resistant genotypes infested with feeding whiteflies, and compare these to the previously obtained profiles. Differences in the proteic activity would be established for the presence of the whitefly as well as the interaction with genotype resistance.

- Determine, through immunodetection, what proteins associated with each genotypes are found in the whiteflies feeding on them. This would provide a direct relationship with the proteic activity in whitefly resistant genotypes.
- Perform SDS-PAGE preparations of total proteins of the genotypes and whiteflies, especially where differences in the initial proteic profiles have been detected and corroborated through immunodetection; thereby extracting different bands, concentrating them in a gel and carry out an electro blot on the membrane.
- Once the electro blot is conducted on the membrane, digestion of the fixed bands in the membrane will be done, with the objective of obtaining internal fragments from the membrane.
- The next step will consist of high resolution electrophoresis of the eluted digestion from the membrane. From here, sequenciation of the amino acids blocked on the N terminal will be carried out and a search for analogues in the amino acid data bank will be performed to determine the protein group, or the type of protein, of selected bands.
- Lastly, a genetic sequence of the protein bands will be done to determine the codifying gene(s).

Activity 4.5. Wild Manihot species as a source of resistance to the cassava whitefly (A*leurotrachelus socialis)*.

Contributors: B. Arias, G. Pérez, C. Ñañez and A. C. Bellotti.

Highlight:

- Numerous genotypes from interspecific crosses with *Manihot esculenta* and wild*Manihot* species, indicating the possible presence of resistance of these pests *in M. flabellifolia*, *M. peruvians*, *M. tristis* and others.

Rationale

Moderate levels of resistance to the cassava whitefly, A*leurotrachelus socialis,* have been identified in *Manihot esculenta* germplasm. More than 90% of the *M. esculenta* accessions in the CIAT cassava germplasm bank have know been evaluated and it is estimated that only about 1% of these have low to moderate levels of resistance to *A. socialis.* These studies have led to the development of a whitefly resistant commercial cultivar, Natiama-31, that has been released to cassava producers by the Colombian Ministry of Agriculture.

The rapid increase and spread of whitefly populations and damage (reduction in cassava yields) necessitates a continued effort to identify additional and better sources of resistance to whiteflies. Recent evaluations of the *Wild Manihot* species in the CIAT collection indicate that high levels of resistance to *A. socialis* may be available in certain species (CIAT Project IP-3, 2003 Annual Report).

CIAT cassava geneticists are producing a considerable number of interspecific crosses between Wild *Manihot* species and *Manihot esculenta*. The cassava entomology section evaluates the progeny from these interspecific crosses for resistance/susceptibility to certain pests, especially mites and whiteflies. The following is a condensed report on some of these evaluations for resistance to the whitefly, *A. socialis.*

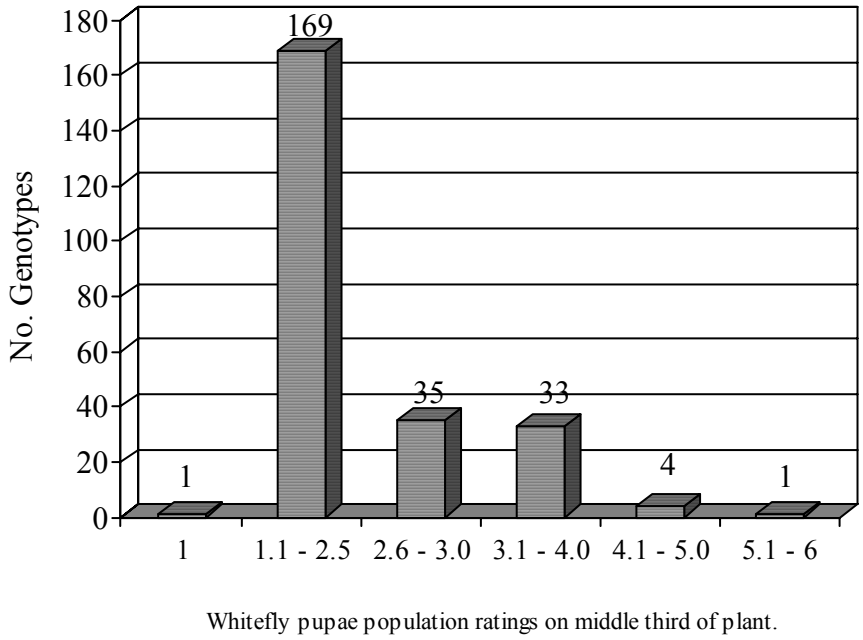


Figure 4.5.1. Whitefly (*A. socialis)* pupae population ratings (1 to 6 scale) on progeny from interspecific crosses (CW) (Dry matter content) at CIAT, Palmira, 2005.

Materials and Methods

Whitefly (*A. socialis)* population and damage evaluations were carried out in seven trials, consisting of interspecific crosses at the CIAT, Palmira (Valle) farm. Numerous *Wild Manihot* species were used in producing the progenyevaluated in these trials. These included *Manihot flabellifolia, M. peruvianus, M. tristis, M.crassesepala* and *M. walkerii.* These wild species were crossed with several *M. esculenta* genotypes, producing more than 1000 interspecific progeny. Each group of crosses designed by the cassava geneticist was being evaluated for specific agronomic traits. These included dry matter content, yellow (high beta-carotene) roots, mite resistance, and polycrosses.

Whitefly population evaluations carried out using a 1 (no whitefly life stages present) to 6 (high populations, often exceeding 5000 individuals per leaf) scale. Plant damage was also based on a 1 (no damage symptoms) to 6 (severe damage; leaf curling, mottled, considerable leaf necrosis and defoliation, sooty mold present) scale. Plant populations and experimental design varied between trials (for details see genetics section).

Results and Discussion

Trial 1. Evaluation of 243 progeny of interspecific crosses designed for high dry matter content. 170 (70%) of the 243 genotypes had a population rating of 1.0 to 2.5 (Figure 4.5.1). Population data was tabulated based on the pupae population of *A. socialis* on the leaves of the middle third of the plant. 23 genotypes had pupae populations rated between 1.0 (CW-14-11) and 1.5 (CW 120-41, CW 121-2, CW 150-30, CW 159-1, CW 164-12 and others). 29% (68) of the genotypes had pupae population ratings

between 2.6 and 4 and only five genotypes had a rating above 4.0 (Figure 4.5.1). Plant damage ratings were correspondingly low as 164 (67.5%) had a leaf damage rating of 1.0. These results indicate either very low whitefly populations, and therefore very low selection pressure, or there is considerably high levels of whitefly resistance in these interspecific genotypes.

Trial 2. Evaluation of 120 progeny of intespecific crosses developed for high dry matter and yellow (high beta-carotene) roots. In this trial 120 CW genotypes were planted in 2 to 4 replications of 10 plant plots (total of 400 plots). *A. socialis* populations were recorded on all the genotypes (Figure 4.5.2). Forty-seven (39.2%) of the genotypes had a low population rating of 1.5 to 2.5 and 61 (51%) had a rating of 2.6 to 3.0, while the remaining 12 genotypes were between 3.1 and 4.0. *M. peruvianus* and *M. tristis* were the *Manihot* species included in many of these crosses. Leaf damage symptoms were absent in 53% (64) of the genotypes, again indicating either high levels of whitefly resistance or low populations.

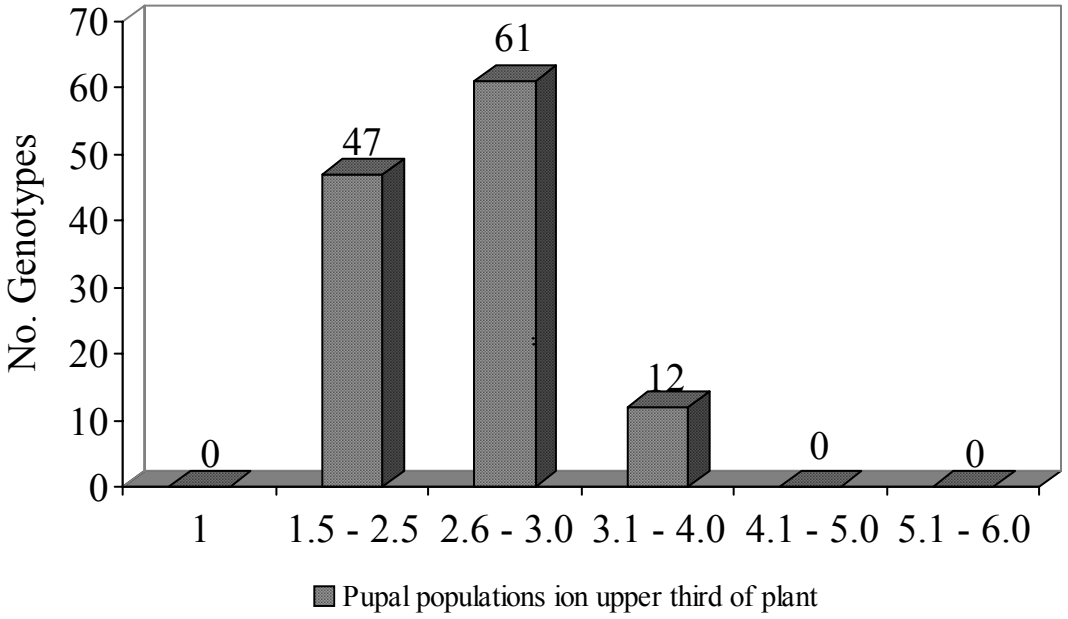


Figure 4.5.2. Whitefly (*A. socialis)* pupae population ratings (1 to 6 scale) on progeny from interspecific crosses (CW: Dry matter content and yellow roots) at CIAT, Palmira, 2005.

Trial 3. Evaluation of 137 progeny of interspecific crosses WWW-PTR-WWW designed for dry matter content. This trial consisted of 137 WWW genotypes sown in 324 plots in three replications. High pupal populations occurred on the upper and middle thirds of the plants, with few lower leaves observed (Figure 4.5.3). Pupae populations were similar for these two levels of the genotypes. In general, whitefly (*A. socialis)* populations were low and only one genotype had a population rating above 4.0. Seven genotypes had no pupae stages on the upper nor middle leaves; these include the genotypes WW 11-18, WW 11-13, WW 11-45, WW 11-6, WW 25-71, WW 30-38 and WW 32-241. In an additional 29 genotypes (21.2%) the pupae population rating did not exceed 1.5 on either the upper or mid leaves. In total 117 genotypes (85.4%) had a maximum pupae rating of 2.5 or lower on the upper or mid leaves. These results indicate high levels of *A. socialis* resistance or very low selection pressure.

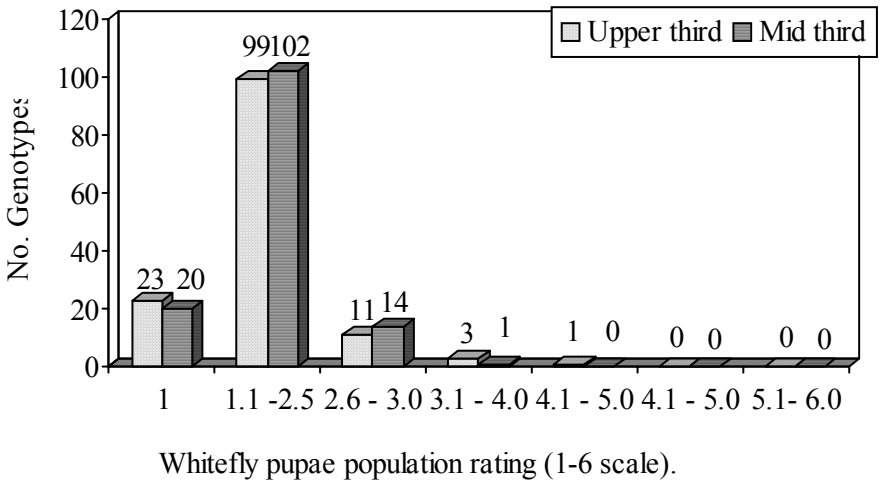


Figure 4.5.3. Whitefly (*A. socialis*) pupae population ratings (1 to 6 scale) on progeny from interspecific crosses (WWW-PRT-WWW) at CIAT, Palmira, 2005.

Trial 4. Evaluation of 330 progeny from interspecific crosses with *M. esculenta* x *Manihot flabelifolia* designed for mite (*Mononychellus tanajoa*) resistance. The 330 genotypes were sown in 10 plant plots with three replicates. Whitefly populations in this trial were low as 35 progeny (10.6%) had no (1.0) pupae on leaves and 287 genotypes (87%) were rated between 1.1 and 2.5 (Figure 4.5.4). Genotypes with no pupae included CW 213-1, CW 217-12, CW 218-7, CW 224-7, CW 225-29, CW 229-25, CW 232-12 and CW 235-2. Leaf damage was absent on 99% of the genotypes, with only one genotype with a 3.0 damage rating.

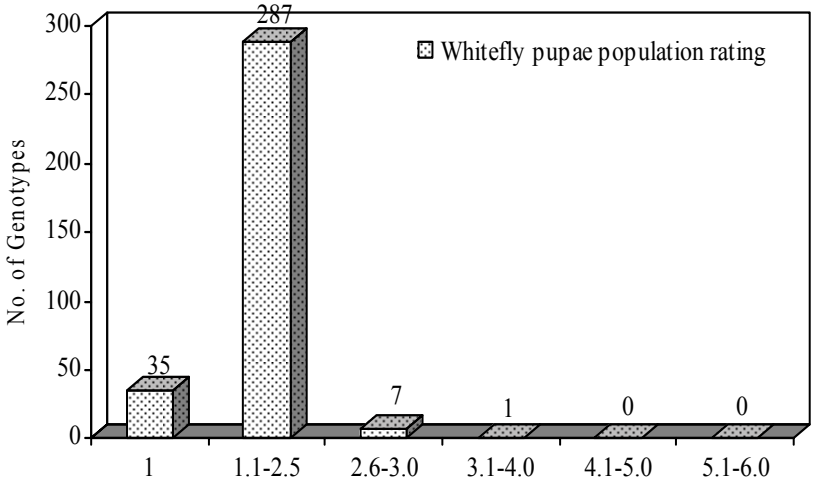


Figure 4.5.4. Whitefly (*A. socialis*) pupae population rating (1 to 6 scale) on progeny from interpsecific cross with *M. flabelifolia* (mite resistente) at CIAT, Palmira, 2005.

Trial 5. Evaluation of 50 progeny from polycrosses for whitefly (*A. socialis)* populations and damage. The polycrosses in this trial involved several *Wild Manihot* species, including *M. flabelifolia, M. tristis, M. peruviana, M. crassisepala and M. walkerii.* The 50 genotypes consisted of 37 CW, 10 OW, 1 CRA, 1 CWR and 1 not identified. *A. socialis* pressure was higher in this trial and whitefly populations and damage ranged from 1.0 to 5.5 on the 1 to 6 rating scales (Figure 4.5.5). Fifteen of the 50 genotypes (30%) had no leaf damage, a 1.0 rating, while 11 genotypes (22%) were rated 1.1 to 2.5 and 20 genotypes (40%) had a susceptible damage rating above 3.0. Whitefly populations were high with a 3.1 to 5.5 rating on 28 genotypes (56%).

Two genotypes, CRA-013 and CW 184-4, had no whiteflies present and 14 additional genotypes had a rating of 1.1 to 2.5. Additional genotypes with low pupae populations and low damage (1.0) ratings include CW 67-89, CW 67-142, CW 67-130, CW 67-44 and CW 67-33. Given the increased selection pressure in this trial, those genotypes with low whitefly populations and low damage ratings should be further evaluated.

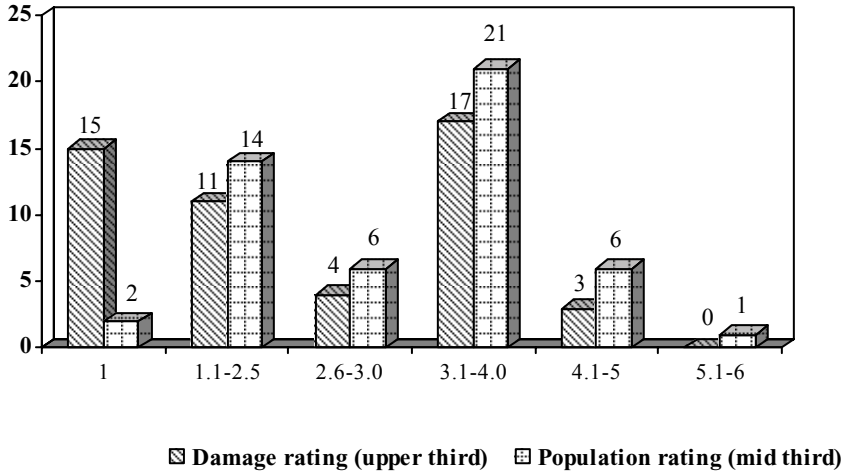


Figure 4.5.5. Whitefly (*A. socialis*) population and damage ratings (1 to 6 scales) on CW and OW polycrosses with several Wild Manihot species (CIAT,

Complete whitefly (*A. socialis)* population and damage evaluations for every genotype in the five trials cited in this report are available in the cassava entomology data files.

Activity 4.6. Establishment of an IPM strategy for whiteflies on cassava in the Colombian Departments of Quindio and Risaralda

Contributors: C. Ma. Holguín, C. J. Herrera and A. C. Bellotti.

Highlight:

- Cassava farmer surveys in Quindío Department of Colombia identify whiteflies as the major pest effecting production. More than 50% of farmers apply chemical pesticides for whitefly control. A whitefly 1PM Project has been initiated.

Rationale

Cassava is traditionally grown on small scale farming systems using few purchased inputs such as fertilizers or pesticides and where cassava is usually one of several crops grown. Distances between cassava fields may be considerable and this can contribute to the sporadic occurrence of some cassava pests. However, in Latin America there are indications of a shift towards larger-scale production units where cassava is grown as a plantation crop. In these situations where cassava is utilized more as an industrial crop, it is advantageous for farmers to employ a multiple planting, multiple harvesting production system in order to meet the constant market demands of the processing industries.

In this type of production system, the cassava crop can be observed at several different growth stages in the same or surrounding farmer fields. Evidence now indicates that pest problems will be compounded in these overlapping production systems. Populations of certain pests, such whiteflies and hornworms, and possibly mealybugs, tend to increase when a constant food supply, e.g. young cassava foliage is available.

The continual food supply prevents or deters a break in the reproductive cycle of the insect, altering its population dynamics that could have been adversely affected by the lack of optimal foliage for feeding and reproduction. This situation is more apt to occur where environmental conditions such as an evenly dispersed rainfall pattern favors or provides for several planting dates throughout a one-year cycle.

In addition, crop management alternatives can be influenced where the availability of irrigation provides for more frequent plantings, especially in semi-arid or seasonally dry agroecosystems.

The aforementioned described conditions occur in certain cassava growing regions of Colombia, Venezuela and Brazil. In the coffee growing region of Colombia, rainfall is dispersed throughout the year and a prolonged dry period of three or more months is not common. In Northeast Brazil (e.g. Bahia State) the availability of irrigation is resulting in large cassava plantations, some more than 3000 ha. In this seasonally dry region cassava was seldom planted more than twice during the year. The availability of irrigation had led to more frequent plantings (Osmar Lorenzi, personnel communication, 2005). A similar situation also occurs in the plains region of Venezuela. The Colombian coffee growing region (CCGR) and Northeast Brasil are reporting more frequent outbreaks of hornworm and increasing whitefly populations.

Whitefly populations in the CCGR have increased dramatically in recent years. Two whitefly species predominate, A*leurotrachelus socialis* and *Trialeurodes variabilis*. This senario has led to the implementation of an IPM project to generate alternatives for whitefly control. Chemical pesticide applications in the region are on the rise and this is increasing production costs as well as the potential to cause outbreaks of secondary pests. Whiteflies are difficult to control with agrochemicals and this

often leads to more frequent applications (on a calendar basis), a build up of pest resistance to the chemical insecticides, and eventually environmental contamination.

The general objective of this project is to establish a whitefly pest management system that will provide cassava producers with an adequate, opportune, economical and sustainable alternative that will reduce pesticide applications and provide good yields.

Specific objectives include:

1) Obtain information on current farmer practices being used by cassava producers.
2) Provide cassava producers with information and training in integrated pest management and provide alternative techniques, such as biological control, reducing pesticide use.

Materials and Methods

A diagnostic survey was conducted with cassava farmers in the target region, in order to obtain information on pest distribution, crop damage levels and present farmer practices being employed to control whiteflies. Thirty cassava farmers were visited and interviewed in the region, especially in the Quindio Department. A survey questionnaire was designed for use during farm visits. These visits and interviews were designed with the purpose of obtaining information on the actual situation confronting cassava producers in the field, the severity of phytosanitary problems and farmer needs and priorities. Posterior to these surveys, random sampling was carried out in cassava fields to confirm the presence of the different insect pests and diseases and to try to determine pest populations or crop damage severity. This information is being tabulated and analyzed.

At the time of this survey meetings were held with farmer groups, students, technicians and agronomists in the region, providing them with information on pest biology and behavior and introducing some whitefly management practices.

Results and Discussion

Surveys were carried out with the assistance of entities such as the Federación Nacional de Cafeteros (The Colombian Coffee Federation) and directly with the Coffee Growers Committee's in each municipality) and ICA Regional Quindio. Cassava producers from six municipalities in the Departments of Quindio (Armenia, Montenegro, Calarcá, Buena Vista, La Tebaida, Quimbaya and Circacia, where cassava is produced at altitudes varying from 1100 to 2900 masl (Figure 4.6.1).

Several pest species were detected feeding on cassava in this region. Whiteflies were the most important and predominant pest found damaging cassava production. Whiteflies were the main pest on 52% of the farms surveyed followed by fruitflies (*Anastrepha sp*.) (20%) and mites (16%) (Figure 4.6.2).

Additional arthropod pests detected in cassava fields included the cassava hornworm (*Erinnyis ello*), whitegrubs, thrips (*Frankliniella williamsi*), the burrower bug (*Cyrtomenus bergi*) and mites (*Mononychelus tanajoa*).

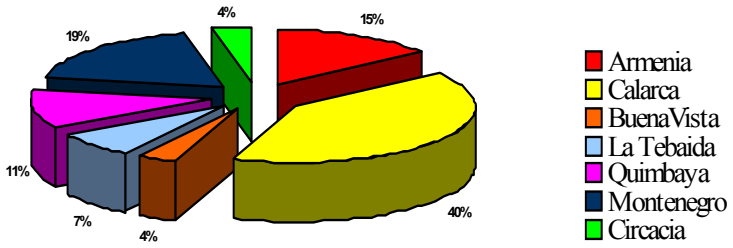


Figure 4.6.1. Municipalities in Quindio Department of Colombia where farmer surveys were conducted to determine cassava management practices. Figures indicate the percentage of the total number of farmers interviewed.

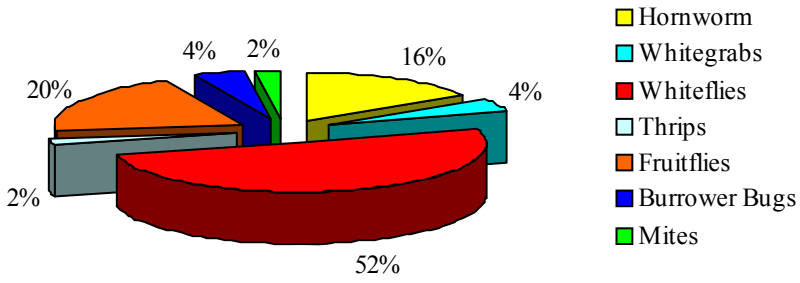


Figure 4.6.2. Arthropod pest populations on cassava farms in Quindío Municipality of Colombia. Figures indicate the percent of farms surveyed with each pest.

At the time of the survey, the municipality of Calarcá was most affected by whiteflies in cassava. This is reflected in the number of cassava farmers that was visited in this municipality (40% of the total). Two species of whiteflies were collected feeding on cassava in the region, A*leurotrachelus socialis* and *Trialeurodes variabilis* (Figure 4.6.3).

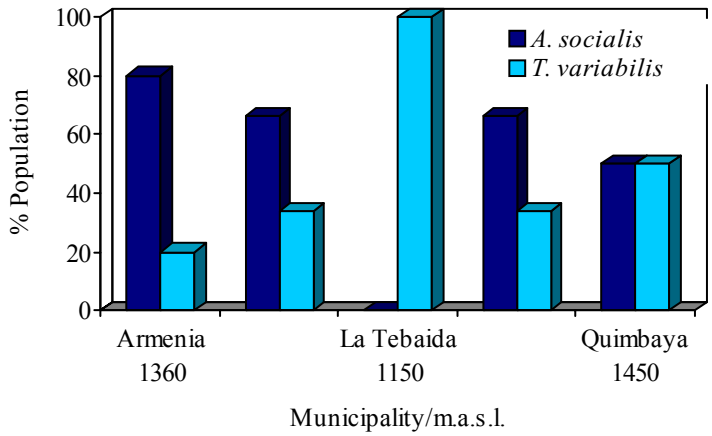


Figure 4.6.3. Percent whitefly species population found on cassava in five municipalities of Quindío Department in Colombia.

At the time of the survey, A. *socialis* was found in higher populations between 1360 and 1780 m.a.s.l., while *T. variabilis* was primarily found on cassava grown between 1100 and 1200 m.a.s.l. This is represented by the municipality of La Tebaida, where ambient temperatures are slightly higher than in the other municipalities in the survey.

The two most frequently grown cassava varieties in the region are Chirosa (MCol 2066) and ICA (HMC-1 and Catumare). Cassava is planted more frequently in monoculture (53.7%) than in association with other crop (46.3%) (Figure 4.6.4).

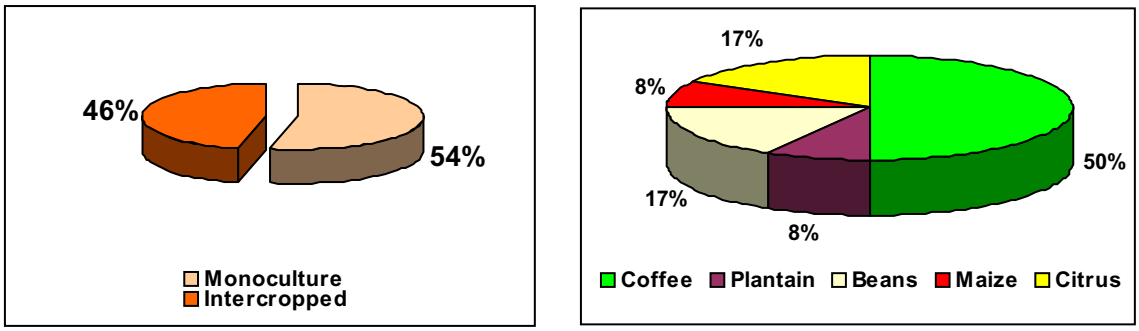


Figure 4.6.4. Cassava cropping systems being employed in Quindío Department of Colombia; a) monoculture vs. intercropping and b) percent of intercropped species.

The crops most frequently grown in association with cassava are coffee (50.0%), beans (17,0%), citrus (17.0%), plantain (8.0%) (Figure 4.6.4). It can also be noted that the whitefly species A. *socialis* predominated in the monoculture plantings of cassava while in the intercropping system the incidence of both species was nearly equal (Figure 4.6.5).

The damage or yield losses in cassava due to whitefly feeding in this region is being determined. Yield loses due to A. *socialis* feeding on cassava in other regions of Colombia have been recorded above 70% and averaging around 30 to 40%

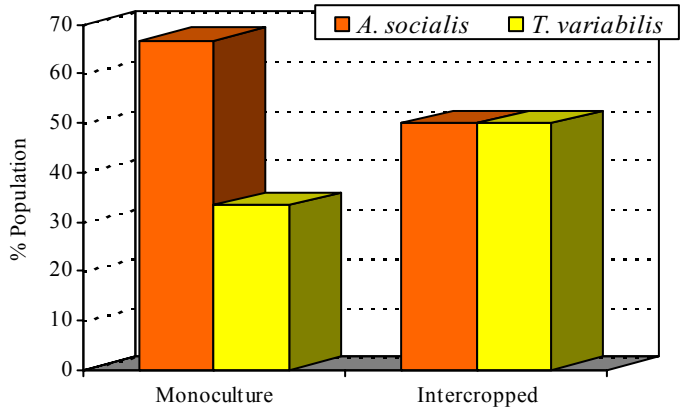


Figure 4.6.5. Percent whitefly species populations in two cassava cropping system, monoculture and intercropped, in Quindío Department of Colombia

The surveys also provided information on cassava farmer knowledge about pest biology, behavior, damage and ultimately about management of cassava pests. Results show that a great majority of producers, approximately 88% have little or no knowledge of whitefly biology, behavior and management. Only 12% of the farmers surveyed claimed knowledge of whiteflies as a cassava pest. These results support the need for farmer training in recognizing cassava pest problems.

Additional survey data shows that at present 50% of the cassava producers are applying chemical insecticides for whitefly control (Figure 4.6.6).

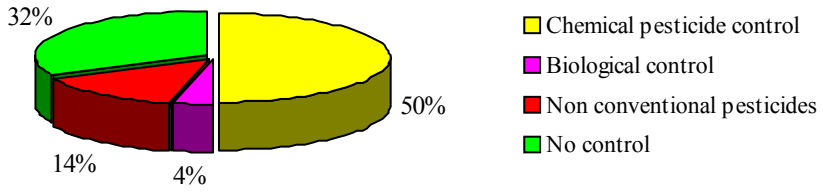


Figure 4.6.6. Whitefly control methods being used by cassava growers in Quindío Department of Colombia.

In most cases they receive no technical training or support. The continued high whitefly populations indicate that these pesticide applications are not effective in controlling the pest. Only 4% are presently using biological control, mostly entomopathogens such as *Beauveria bassiana* and *Lecanicillium lecanni* and predators like Crysopa. Approximately 14% of the farmers are using non-conventional pesticides; these are "home-remedy" types such as plant extracts and soap solutions. 32% of the farmers are not engaged in any whitefly control.

Cassava farmers are presently applying several different chemical pesticide products for whitefly control. The most popular is Sistemin (Dimethoate), being applied by nearly 48% of the farmers. Second is Actara (Tiametoxan) used by 19% of the growers, followed by Nodrin (9.5%) and Evisect (9.5%) (Table 4.6.1).

Products such Eviset (Tyocicam), Actara (Tiametoxan) and Trebon (Etoferprox) are reported as providing efficient control of whiteflies. However, farmers are not achieving adequate whitefly control, probably due to their lack of knowledge of whitefly biology and behavior. Pesticide applications are not timed to coincide with whitefly adult and 1st instar nymphal populations when these species are most susceptible to chemical treatment.

Similar surveys of cassava farmers in the Departments of Risaralda and Caldas have already been initiated as the project will be expanded into these two regions. Chemical pesticide and non-conventional pesticide products are being evaluated on farmer fields in the region to determine product efficiency and mode and timing of applications. Several biological control options will be evaluated. These include the use of entomopathogens and predator or parasite species. A whitefly resistant cassava variety is being compared to farmer varieties for acceptance as part of the varietal mixture.

The need to have farmers more knowledgeable about whitefly biology and behavior is obvious. Information sharing with growers has been initiated and courses in whitefly IPM have already been implemented. To date approximately 600 farmers and technicians have participated in these workshops and seminars.

Table 4.6.1. Percent usage by cassava growers of chemical pesticide products for cassava whitefly control in Quindío Department of Colombia.

Product/Activities Ingredient	% Utilization
Nodrin (Methomyl)	9.5
Sistemin (Dimetoato)	47.6
Evisect (Tyociclam)	9.5
Actara (Tiametoxam)	19.0
Thionil (Endosulfan)	4.8
Trebon (Etoferprox)	4.8

Activity 4.7. Intrinsic rate of increase the whitefly *Aleurotrachelus socialis* on the cassava (*Manihot esculenta)* varieties CMC-40 and MCol 2066 (Chirosa).

Contributors: C. Ma. Holguín, A. Carabali & A. C. Bellotti.

Highlight:

- The high rate of survival and short generation time of the cassava whitefly, *Aleurotrachelus socialis* feeding on the commercial variety Chirosa, explain its rapid population increase in cassava plantations.

Rationale

Whiteflies as direct feeding pests constitute a major problem in cassava production in Central and South America and the Caribbean Region. There is a large complex in the neotropics where 11 species are reported. The major species causing yield losses in the northern region of South America (Colombia, Venezuela and Ecuador) and certain areas of Central America, is *Aleurotrachelus socialis.* On experimental fields, infestations of 1 month resulted in a 5% yield reduction of 6 months in a 42% reduction, and of 11 months in a 79% reduction (Vargas and Bellotti,1981, Revista Columbiana de Entomología 7: 13-20).

In recent years whitefly populations, especially of *A. socialis* have increased dramatically in certain cassava growing regions of Colombia. These include the Departments of Cauca, Valle del Cauca and the Colombian coffee growing region of Quindio, Risaralda and Caldas. In this latter region, prior to about three years ago, whitefly populations were relatively low and not suspected of causing yield losses. The rapid increase in whitefly populations, principally *A.socialis,* but also *Trialeurodes variabilis*, is presently causing severe crop damage.

The reason for this rapid buildup in populations is not fully understood. *A. socialis* is more trypically associated with lower altitudes and warmer temperatures as found in the Tolima Valley where the pest has been endemic for many years.

Studies have been initiated to try to better comprehend the possible changes in *A. socialis* biology and parameter population. These studies can provide information on the potential of *A. socialis* to invade

different cassava growing regions and will aid in developing effective pest management programs. The immediate objective of this research is to determine the biology of *A. socialis* and estimate population parameters on two cassava genotypes.

Materials and Methods

Two cassava genotypes were used in the experimental trials. The genotype CMC-40 is a vigorous cultivar that is susceptible to whiteflies; Chirosa (MCol 2066) is a high yielding commercial cultivar being planted throughout the coffee growing region of Colombia. Experiments are being carried out on 30 to 40 day plants being grown in plastic pots with sterile soil (1.0 kg). Plants are maintained in the screenhouse at 30 $\pm$ 2°C and RH of 50 to 60%.

A. *socialis* adults are obtained from the CIAT colony that is maintained in the greenhouse (27 $\pm$ °C temp. and 60 – 70% RH), on the cassava cultivar CMC-40.

Longevity and Fecundity Studies: Forty recently emerged A*leurotrachelus socialis* females, sexed using the Eichelkrant Cardona (1989, Turrialba 39:55-62) method, are selected from the CIAT colony. These females are placed individually in small leaf cages (2.5 cm diameter x 2.0 cm width) and placed on the undersides of the test genotypes, Chirosa and CMC-40. Every 48 hours adults were transferred to a new area of the leaf; this procedure is continued until the natural death of the whitefly females. Fecundity was estimated by recording the numbers of eggs oviposited by each female during these 48 hours intervals. Adult longevity was determined by the duration of survival of each female.

Development time, rate of survival and proportional females: Fifty two day old adult male and female, A. *socialis* were removed from the CIAT colony with the aid of a bucal aspirator. These were placed in the small leaf cages, as previously described, and attached to the undersides of CMC-40 and Chirosa leaves. Adults were removed after six hours and 200 eggs were randomly selected. Egg to adult development time was recorded, as well as the rate of survival of immature stages and the proportion if emerging females.

Demographic Parameters: Data on development time is combined with experimental data on reproduction (1xmx); generating life tables which are used to calculate the demographic parameters as defined by Price (1975, Insect Ecology, John Wiley & Sons, New York, 514 p): 1) Net reproduction rate ($_{RO}$), the average number of female descendents produced by one female per generation; 2) generation time (T), equivalent to the time period between parental birth and progeny birth and 3), the intrinsic rate of population increase (rm) estimated using the equation:

$$\times \exp(-rmX)1_x m_x = 1$$

Where X is the female age (days); 1 x is specific survival age and m_x represents the proportion of females from a female progeny at age X. To calculate the value of rm, the corrected age X + 0.5 and the equation In 2/rm were use to estimate the days required to double the population.

Results and Discussion

Longevity and Fecundity of Aleurotrachelus socialis: Longevity of A. *socialis* feeding on MCol 2066 (Chirosa) ranged from 2 to 8 days while the range on CMC-40 was 2 to 16 days; the average longevity of *A. socialis* on CMC-40 exceed that on MCol 2066 by 2.5 days (Table 4.7.1).

Table 4.7.1. Average longevity (days), average fecundity (eggs/female) and oviposition rate (eggs/females/2 days) of *Aleurotrachelus socialis* feeding on two cassava genotypes, CMC-40 and MCol 2066 (Chirosa).

Parameter	CMC-40	Chirosa (Mcol 2066)
Average Longevity (days)	6.4 a	3.9 b
Range	2 – 16	2 - 8
Average fecundity	33.8 a	7 b
Range	1 - 125	1 - 154
Average oviposition rate	5.29 a	1.8 b

Averages followed by different letters across the columns are significantly different λ ANOVA – one way P{ 0.0001, Tukey P{ 0.05.

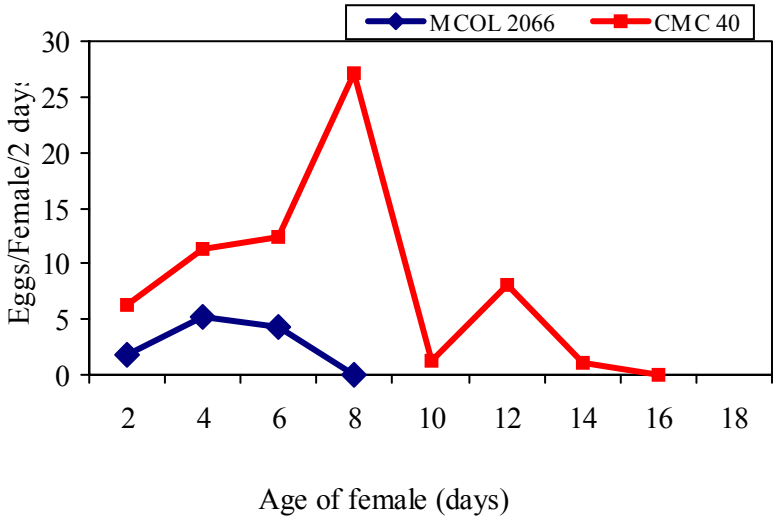


Figure 4.7.1. Fecundity of *A.socialis* feeding on cassava genotypes CMC-40 and MCol 2066 in the secreenhouse.

Ovipositional range per female was greater on CMC-40 (1-125) than that expressed on MCol 2006) 1-54) (Table 4.7.1). Average fecundity was significantly higher on CMC-40 than on MCol 2066. Peak oviposition per female on CMC-40 occurred on day 8, while oviposition on Mcol 2066 terminated during this period (Figure 4.7.1). Average fecundity on CMC-40 was 33.8 eggs per female and only 7.0 on MCol 2006. Ovipositional rate was higher on CMC-40, where each female oviposited 3.5 more eggs every two days, than on MCol 2066 (Table 4.7.1).

Development time, rate of survival of immature stages and proportion of females: The development time of *A. socialis* feeding on CMC-40 was significantly longer by 5 days than those feeding on MCol 2066 (Table 4.7.2).

Table 4.7.2. Development time, survival and proportions of female *Aleurotrachelus socialis* feeding on two cassava genotypes CMC-40 and MCol 2066 (Chirosa)

Parameter	CMC – 40	Chirosa MCol 2066
Development time (days)	39.7 b	34.7 a
Survival rate (%)	80 (160)	87 (174)
Proportional females (%)	50	50

Development time: different letters between columns are significantly different ANOVA Tukey test P{ 0.05. Rate of survival of immatures (χ^2=3.56, 1 df, P= 0.0593)

There was no significant difference in rate of survival between the two genotypes although survival was higher on MCol 2066 (174 vs. 160). These results indicate that *A.socialis* will successfully colonize both genotypes. The proportion of females emerging was the same on both genotypes, 1:1 (Table 4.7.2).

Demographic Parameters: The net reproductive rate (Rŭ allows us to estimate that, on average, at the end of a generation, *A. socialis* populations could multiply 33.7 times (individual/individual) on CMC-40, this being 27.5 times greater than on MCol 2066 (Table 4.7.3).

Table 4.7.3. Demographic parameters of *Aleurotrachelus socialis* feeding on CMC-40 and MCol 2066 (Chirosa)

Parameter	CMC-40	Chirosa (Mcol 2066)
Net Reproduction Rate (Ro)	33.74	6.2
Generation time (T)	44.23	36.71
Intrinsic rate of increase (r_m)	0.0296	0.0495
Days to duplicate population (DDP In $2r_m$	23.4	14

This considerable difference can be attributed to the low fecundity level of *A. socialis* on MCol 2066. *A. socialis* would complete one generation in 44.2 days on CMC-40 and in 36.7 days on Mcol 2066. This indicates that *A. socialis* would complete 10 generations per year on CMC-40 and 12 MCol 2066.

The intrinsic rate of increase (rm) was 40% greater on MCol 2066, when compared to CMC-40 (Table 4.7.3). These results demonstrate the biotic potential of *A. socialis* to develop high populations on Mcol 2066 (Chirosa), inspite of the lower fecundity that occurs on this host. *A. socialis* will double its population in 14 days feeding on MCol 2066, while it requires 23 days on CMC-40 (Table 4.7.3).

It can be conclude from the results of this research that both CMC-40 and Chirosa are favorable host genotypes for rapid population increases of *A. socialis.* The shorter development time and the high rate of survival of *A.socialis* on Chirosa (MCol 2066) are indicators for high whitefly populations found on this genotype. These results help explain the high populations and damage to the cassava crop being experienced in the Colombian coffee growing region (Figure 4.7.2).

Figure 4.7.2. High survival of populations of *Aleurotrachelus socialis* feeding on Chirosa (MCol 2066) (a) and CMC-40 (b).

Annexes

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